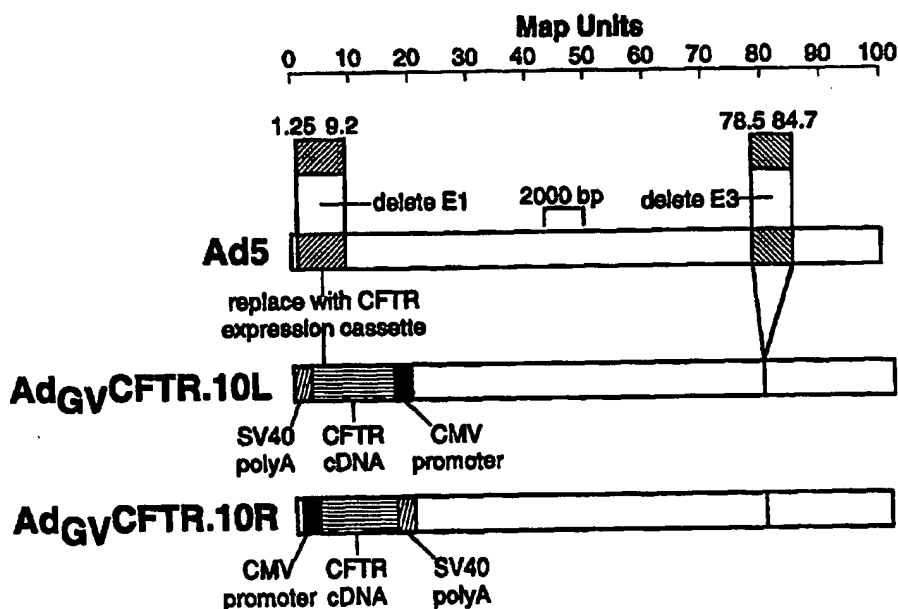




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/86, C07K 14/075, C12N 15/34, A61K 48/00, C07K 14/47, C12N 5/10, 7/01, 15/12		A1	(11) International Publication Number: WO 95/34671
			(43) International Publication Date: 21 December 1995 (21.12.95)
(21) International Application Number: PCT/US95/07341		[US/US]; 5329 Randolph Road, Rockville, MD 20852 (US).	
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(30) Priority Data: 258,416 10 June 1994 (10.06.94) US		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).	
(60) Parent Application or Grant (63) Related by Continuation US 08/258,416 (CIP) Filed on 10 June 1994 (10.06.94)		Published With international search report.	
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(54) Title: COMPLEMENTARY ADENOVIRAL VECTOR SYSTEMS AND CELL LINES



(57) Abstract

The present invention provides multiply deficient adenoviral vectors and complementing cell lines. Also provided are recombinants of the multiply deficient adenoviral vectors and a therapeutic method, particularly relating to gene therapy, vaccination, and the like, involving the use of such recombinants.

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COMPLEMENTARY ADENOVIRAL VECTOR SYSTEMS AND CELL LINES

RELATED APPLICATION

This is a continuation-in-part application of
compending U.S. patent application Serial No. 08/258,416,
5 filed June 10, 1994.

Technical Field of the Invention

The present invention relates to recombinant,
multiply deficient adenoviral vectors and to
complementing cell lines useful in the culturing of such
10 vectors. In addition, the present invention relates to
the therapeutic use of such vectors.

Background of the Invention

During the winter and spring of 1952-1953, Rowe and
his colleagues at the National Institutes of Health (NIH)
15 obtained and placed in tissue culture adenoids that had
been surgically removed from young children in the
Washington, D.C. area (Rowe et al., Proc. Soc. Exp. Biol.
Med., 84, 570-573 (1953)). After periods of several
weeks, many of the cultures began to show progressive
20 degeneration characterized by destruction of epithelial
cells. This cytopathic effect could be serially
transmitted by filtered culture fluids to established
tissue cultures of human cell lines. The cytopathic
agent was called the "adenoid degenerating" (Ad) agent.
25 The name "adenovirus" eventually became common for these
agents. The discovery of many prototype strains of
adenovirus, some of which caused respiratory illnesses,
followed these initial discoveries (Rowe et al., supra;
Dingle et al., Am. Rev. Respir. Dis., 97, 1-65 (1968);
30 reviewed in Horwitz, "Adenoviridae and Their
Replication," in Virology (Fields et al., eds., Raven
Press Ltd., New York, NY, 2d ed., 1990), pp. 1679-1721).

Over 40 adenoviral subtypes have been isolated from humans and over 50 additional subtypes have been isolated from other mammals and birds (reviewed in Ishibashi et al., "Adenoviruses of animals," in The Adenoviruses, 5 Ginsberg, ed., Plenum Press, New York, NY, pp. 497-562 (1984); Strauss, "Adenovirus infections in humans," in The Adenoviruses, Ginsberg, ed., Plenum Press, New York, NY, pp. 451-596 (1984)). All these subtypes belong to the family Adenoviridae, which is currently divided into 10 two genera, namely Mastadenovirus and Aviadenovirus.

All adenoviruses are morphologically and structurally similar. In humans, however, adenoviruses have diverging immunological properties and, therefore, are divided into serotypes. Two human serotypes of 15 adenovirus, namely Ad2 and Ad5, have been studied intensively. These studies have provided the majority of information about adenoviruses in general.

Adenoviruses are nonenveloped, regular icosahedrons, 65-80 nm in diameter, consisting of an external capsid 20 and an internal core. The capsid is composed of 20 triangular surfaces or facets and 12 vertices (Horne et al., J. Mol. Biol., 1, 84-86 (1959)). The facets are comprised of hexons and the vertices are comprised of pentons. A fiber projects from each of the vertices. In 25 addition to the hexons, pentons, and fibers, there are eight minor structural polypeptides, the exact positions of the majority of which are unclear. One minor polypeptide component, namely polypeptide IX, binds at positions where it can stabilize hexon-hexon contacts at 30 what is referred to as the group-of-nine center of each facet (Furcinitti et al., EMBO, 8, 3563-3570 (1989)). The minor polypeptides VI and VIII are believed to stabilize hexon-hexon contacts between adjacent facets. The minor polypeptide IIIA, which is known to be located 35 in the regions of the vertices, has been suggested by

Stewart et al. (Cell, 67, 145-154 (1991)) to link the capsid and the core.

The viral core contains a linear, double-stranded DNA molecule with inverted terminal repeats (ITRs), which
5 have been noted to vary in length from 103 bp to 163 bp in different isolates (Garon et al., Proc. Natl. Acad. Sci. USA, 69, 2391-2394 (1972); Wolfson et al., Proc. Natl. Acad. Sci. USA, 69, 3054-3057 (1972); Arrand et al., J. Mol. Biol., 128, 577-594 (1973); Steenberg et
10 al., Nucleic Acids Res., 4, 4371-4389 (1977); and Tooze, DNA Tumor Viruses, 2nd ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory. pp. 943-1054 (1981)). The ITRs harbor origins of DNA replication (Garon et al., supra; Wolfson et al., supra; Arrand et al., supra;
15 Steenberg et al., supra).

The viral DNA is associated with four polypeptides, namely V, VII, μ , and terminal polypeptide (TP). The 55 kd TP is covalently linked to the 5' ends of the DNA via a dCMP (Rekosh et al., Cell, 11, 283-295 (1977); Robinson
20 et al., Virology, 56, 54-69 (1973)). The other three polypeptides are noncovalently bound to the DNA and fold it in such a way as to fit it into the small volume of the capsid. The DNA appears to be packaged into a structure similar to cellular nucleosomes as seen from
25 nuclease digestion patterns (Corden et al., Proc. Natl. Acad. Sci. USA, 73, 401-404 (1976); Tate et al., Nucleic Acids Res., 6, 2769-2785 (1979); Mirza et al., Biochim. Biophys. Acta, 696, 76-86 (1982)).

The overall organization of the adenoviral genome is
30 conserved among serotypes, such that specific functions are similarly positioned. The Ad2 and Ad5 genomes have been completely sequenced and sequences of selected regions of genomes from other serotypes are available as well.

35 Adenovirus begins to infect a cell by attachment of the fiber to a specific receptor on the cell membrane

(Londberg-Holm et al., J. Virol., 4, 323-338 (1969); Morgan et al., J. Virol., 4, 777-796 (1969); Pastan et al., "Adenovirus entry into cells: some new observations on an old problem," in Concepts in Viral Pathogenesis,
5 Notkins et al., eds., Springer-Verlag, New York, NY, pp. 141-146 (1987)). Then, the penton base binds to an adenoviral integrin receptor. The receptor-bound virus then migrates from the plasma membrane to clathrin-coated pits that form endocytic vesicles or receptosomes, where
10 the Ph drops to 5.5 (Pastan et al., Concepts in Viral Pathogenesis, Notkins and Oldstone, eds. Springer-Verlag, New York. pp. 141-146 (1987)). The drop in Ph is believed to alter the surface configuration of the virus, resulting in receptosome rupture and release of virus
15 into the cytoplasm of the cell. The viral DNA is partially uncoated, i.e., partially freed of associated proteins, in the cytoplasm while being transported to the nucleus.

When the virus reaches the nuclear pores, the viral
20 DNA enters the nucleus, leaving most of the remaining protein behind in the cytoplasm (Philipson et al., J. Virol., 2, 1064-1075 (1968)). However, the viral DNA is not completely protein-free in that at least a portion of the viral DNA is associated with at least four viral
25 polypeptides, namely V, VII, TP and μ , and is converted into a viral DNA-cell histone complex (Tate et al., Nucleic Acids Res., 6, 2769-2785 (1979)).

The cycle from cell infection to production of viral particles lasts 1-2 days and results in the production of
30 up to 10,000 infectious particles per cell (Green et al., Virology, 13, 169-176 (1961)). The infection process of adenovirus is divided into early (E) and late (L) phases, which are separated by viral DNA replication, although some events which take place during the early phase also
35 take place during the late phase and vice versa. Further

subdivisions have been made to fully describe the temporal expression of viral genes.

During the early phase, viral mRNA, which constitutes a minor proportion of the total RNA present in the cell, is synthesized from both strands of the adenoviral DNA present in the cell nucleus. At least five regions, designated E1, E2, E3, E4, and MLP-L1, are transcribed (Lewis et al., Cell, 7, 141-151 (1976); Sharp et al., Virology, 75, 442-456 (1976); Sharp, "Adenovirus transcription," in The Adenoviruses, Ginsberg, ed., Plenum Press, New York, NY, pp. 173-204 (1984)). Each region has at least one distinct promoter and is processed to generate multiple mRNA species.

The products of the early (E) regions (1) serve regulatory roles for the expression of other viral components, (2) are involved in the general shut-off of cellular DNA replication and protein synthesis, and (3) are required for viral DNA replication. The intricate series of events regulating early mRNA transcription begins with expression of certain immediate early regions, including E1A, L1, and the 13.5 kd gene (reviewed in Sharp (1984), supra; Horwitz (1990), supra). Expression of the delayed early regions E1B, E2A, E2B, E3 and E4 is dependent on the E1A gene products. Three promoters, the E2 promoter at 72 map units (mu), the protein IX promoter, and the IVa promoter are enhanced by the onset of DNA replication but are not dependent on it (Wilson et al., Virology, 94, 175-184 (1979)). Their expression characterizes an intermediate phase of viral gene expression. The result of the cascade of early gene expression is the start of viral DNA replication.

Adenoviral DNA replication displaces one parental single-strand by continuous synthesis in the 5' to 3' direction from replication origins at either end of the genome (reviewed in Kelly et al., "Initiation of viral DNA replication," in Advances in Virus Research,

Maramorosch et al., eds., Academic Press, Inc., San Diego, CA, 34, 1-42 (1988); Horwitz (1990), supra; van der Vliet, "Adenovirus DNA replication in vitro," in The Eukaryotic Nucleus, Strauss et al., eds., Telford Press, Caldwell, NJ, 1, 1-29 (1990)). Three viral proteins encoded from E2 are essential for adenoviral DNA synthesis: (1) the single-stranded DNA binding protein (DBP), (2) the adenoviral DNA polymerase (Ad pol), and (3) the pre-terminal protein (pTP). In addition to these essential proteins, in vitro experiments have identified many host cell factors necessary for DNA synthesis.

DNA synthesis is initiated by the covalent attachment of a dCMP molecule to a serine residue of pTP. The pTP-DCMP complex then functions as the primer for Ad pol to elongate. The displaced parental single-strand can form a panhandle structure by base-pairing of the inverted terminal repeats. This terminal duplex structure is identical to the ends of the parental genome and can serve as an origin for the initiation of complementary strand synthesis.

Initiation of viral DNA replication appears to be essential for entry into the late phase. The late phase of viral infection is characterized by the production of large amounts of the viral structural polypeptides and the nonstructural proteins involved in capsid assembly. The major late promoter (MLP) becomes fully active and produces transcripts that originate at 16.5 mu and terminate near the end of the genome. Post-transcriptional processing of this long transcript gives rise to five families of late mRNA, designated respectively as L1 to L5 (Shaw et al., Cell, 22, 905-916 (1980)). The mechanisms that control the shift from the early to late phase and result in such a dramatic shift in transcriptional utilization are unclear. The requirement for DNA replication may be a *cis*-property of the DNA template, because late transcription does not

occur from a superinfecting virus at a time when late transcription of the primary infecting virus is active (Thomas et al., Cell, 22, 523-533 (1980)).

Assembly of the virion is an intricate process from the first step of assembling major structural units from individual polypeptide chains (reviewed in Philipson, "Adenovirus Assembly," in The Adenoviruses, Ginsberg, ed., Plenum Press, New York, NY (1984), pp. 309-337; Horwitz (1990), supra). Hexon, penton base, and fiber assemble into trimeric homopolymer forms after synthesis in the cytoplasm. The 100 kd protein appears to function as a scaffolding protein for hexon trimerization and the resulting hexon trimer is called a hexon capsomer. The hexon capsomeres can self-assemble to form the shell of an empty capsid, and the penton base and fiber trimers can combine to form the penton when the components are inside the nucleus. The facet of the icosahedron is made up of three hexon capsomeres, which can be seen by dissociation of the capsid, but the intermediate step of formation of a group-of-nine hexons has not been observed. Several assembly intermediates have been shown from experiments with temperature-sensitive mutants. The progression of capsid assembly appears dependent on scaffolding proteins, 50 kd and 30 kd. The naked DNA most probably enters the near-completed capsid through an opening at one of the vertices. The last step of the process involves the proteolytic trimming of the precursor polypeptides pVI, pVII, pVIII and pTP, which stabilizes the capsid structure, renders the DNA insensitive to nuclease treatment, and yields a mature virion.

Certain recombinant adenoviral vectors have been used in gene therapy. The use of a recombinant adenoviral vector to transfer one or more recombinant genes enables targeted delivery of the gene or genes to an organ, tissue, or cells in need of treatment, thereby

overcoming the delivery problem encountered in most forms of somatic gene therapy. Furthermore, recombinant adenoviral vectors do not require host cell proliferation for expression of adenoviral proteins (Horwitz et al., in 5 Virology, Raven Press, New York, 2, 1679-1721 (1990); and Berkner, BioTechniques, 6, 616 (1988)). Moreover, if the diseased organ in need of treatment is the lung, use of adenovirus as the vector of genetic information has the added advantage of adenovirus being normally trophic for 10 the respiratory epithelium (Straus, in Adenoviruses, Plenum Press, New York, pp. 451-496 (1984)).

Other advantages of adenoviruses as potential vectors for human gene therapy are as follows:

(i) recombination is rare; (ii) there are no known 15 associations of human malignancies with adenoviral infections despite common human infection with adenoviruses; (iii) the adenoviral genome (which is linear, double-stranded DNA) currently can be manipulated to accommodate foreign genes ranging in size up to 7.0- 20 7.5 kb in length; (iv) an adenoviral vector does not insert its DNA into the chromosome of a cell, so its effect is impermanent and unlikely to interfere with the cell's normal function; (v) the adenovirus can infect non-dividing or terminally differentiated cells, such as 25 cells in the brain and lungs; and (vi) live adenovirus, having as an essential characteristic the ability to replicate, has been safely used as a human vaccine (Horwitz et al., supra; Berkner et al., J. Virol., 61, 1213-1220 (1987); Straus supra; Chanock et al., JAMA, 30 195, 151 (1966); Haj-Ahmad et al., J. Virol., 57, 267 (1986); and Ballay et al., EMBO, 4, 3861 (1985)).

Foreign genes have been inserted into two major regions of the adenoviral genome for use as expression vectors, namely the E1 and E3 regions, thus providing 35 singly deficient adenovirus and vectors derived therefrom. Insertion into the E1 region results in

defective progeny that require either growth in complementary cells or the presence of an intact helper virus, either of which serves to replace the function of the impaired or absent E1 region (Berkner et al., supra;
5 Davidson et al., J. Virol., 61, 1226-1239 (1987); and Mansour et al., Mol. Cell Biol., 6, 2684-2694 (1986)). This region of the genome has been used most frequently for expression of foreign genes.

The genes inserted into the E1 region have been
10 placed under the control of various promoters and most produce large amounts of the foreign gene product, dependent on the expression cassette. These adenoviral vectors, however, will not grow in noncomplementing cell lines. Currently, there are only a few cell lines that
15 exist that will complement for essential functions missing from a singly deficient adenovirus. Examples of such cell lines include HEK-293 (Graham et al., Cold Spring Harbor Symp. Quant. Biol., 39, 637-650 (1975)), W162 (Weinberg et al., Proc. Natl. Acad. Sci. USA, 80,
20 5383-5386 (1983)), and gMDBP (Klessig et al., Mol. Cell Biol., 4, 1354-1362 (1984); Brough et al., Virology, 190, 624-634 (1992)).

The E3 region, the other region noted above as useful for insertion of foreign genes, is nonessential
25 for virus growth in tissue culture, and replacement of this region with a foreign gene expression cassette leads to a virus that can productively grow in a noncomplementing cell line. For example, the insertion and expression of the hepatitis B surface antigen in the
30 E3 region with subsequent inoculation and formation of antibodies in the hamster has been reported (Morin et al., Proc. Natl. Acad. Sci. USA, 84, 4626-4630 (1987)).

The problem with singly deficient adenoviral vectors is that they limit the amount of usable space within the
35 adenoviral genome for insertion and expression of a foreign gene. Due to similarities, or overlap, in the

viral sequences contained within the singly deficient adenoviral vectors and the complementing cell lines that currently exist, recombination events (which as noted above are ordinarily very rare) can take place and create
5 replication competent viruses within a vector stock so propagated. This event can render a stock of vector unusable for gene therapy purposes as a practical matter.

Accordingly, it is an object of the present invention to provide multiply deficient adenoviral
10 vectors that can accommodate insertion and expression of larger pieces of foreign DNA. It is another object of the present invention to provide cell lines that complement the present inventive multiply deficient adenoviral vectors. It is also an object of the present
15 invention to provide recombinants of multiply deficient adenoviral vectors and therapeutic methods, particularly relating to gene therapy, vaccination, and the like, involving the use of such recombinants. These and other objects and advantages of the present invention, as well
20 as additional inventive features, will be apparent from the following detailed description.

Brief Summary of the Invention

The present invention provides multiply deficient adenoviral vectors and complementing cell lines. The
25 multiply deficient adenoviral vectors can accommodate insertion and expression of larger fragments of foreign DNA than is possible with currently available singly deficient adenoviral vectors. The multiply deficient adenoviral vectors are also replication deficient and
30 resistant to recombination when propagated or grown in complementing cell lines, which features are particularly desirable for gene therapy and other therapeutic purposes. Accordingly, the present invention also provides recombinant multiply deficient adenoviral
35 vectors and therapeutic methods, for example, relating to

gene therapy, vaccination, and the like, involving the use of such recombinants.

Brief Description of the Drawings

Figure 1 is a set of schematic diagrams of the
5 Ad_{Gv}CFTR.10L and Ad_{Gv}CFTR.10R viral vectors.

Figure 2 is a set of schematic diagrams of the Ad_{Gv}CFTR.11 viral vectors.

Figure 3 is a schematic diagram of the Ad_{Gv}CFTR.12 viral vector.

10 Figure 4 is a schematic diagram of the Ad_{Gv}CFTR.13 viral vector.

Figure 5 is a schematic diagram of an E2A expression vector.

15 Figure 6 is a representation of an immunoblot used to screen for the level of induced DBP expression in certain clonal 293/E2A cell lines.

Figure 7 is a representation of an immunoblot used to analyze the accumulation of DBP by certain clonal 293/E2A cell lines over the first 24 hours of induction.

20 Figure 8 is a set of schematic diagrams of the Ad_{Gv}CFTR.10L and Ad_{Gv}CFTR.12B viral vectors.

Figure 9 is a reverse contrast photograph of a DNA gel stained with ethidium bromide, and provides data relating to the PCR detection of the Ad_{Gv}CFTR.12B viral
25 vector from passaged transfection lysates.

Figure 10 is a schematic diagram of the Ad_{Gv}Luc;E2GUS viral vector.

Figure 11 is a schematic diagram of an E4-ORF6 expression vector.

30 Figure 12 is a reverse contrast photograph of a DNA gel stained with ethidium bromide, and provides data relating to the PCR detection of the E4 deletion region in passaged lysates of Ad_{Gv}βgal.12.

Figure 13 is a bar graph that depicts the level of PFU/cell (y-axis) per certain cell lines (x-axis) after transfection.

Figure 14 is a schematic diagram of the Ad_{Gv}βgal.12
5 viral vector.

Detailed Description of the Invention

The present invention provides, among other things, multiply deficient adenoviral vectors for gene cloning and expression. The multiply deficient adenoviral
10 vectors of the present invention differ from currently available singly deficient adenoviral vectors in being deficient in at least two regions of the adenoviral genome and in being able to accept and express larger pieces of foreign DNA. The term "foreign DNA" is used
15 herein to refer to any sequence of DNA inserted into a vector of the present invention that is foreign to the adenoviral genome. Such foreign DNA may constitute genes, portions of genes, or any other DNA sequence, including but not limited to sequences that encode RNA,
20 anti-sense RNA, and/or polypeptides.

A region of the adenoviral genome comprises one or more genes. Horwitz (1990), supra. Such genes encode gene products that mediate, facilitate, cause, or are the various components or activities of the adenovirus, such
25 as attachment, penetration, uncoating, replication, core protein, hexon, fiber, hexon associated protein, and the like. One effect of a deficient region can be an inability of the adenovirus to propagate, for example, which may involve any or all of the aforementioned
30 components or activities. The aforementioned components or activities are referred to herein as gene functions.

A deficiency in a gene or gene function, i.e., a deficient gene, gene region, or region, as used herein is defined as a deletion of genetic material of the viral
35 genome, which serves to impair or obliterate the function

of the gene whose DNA sequence was deleted in whole or in part and to provide room in or capacity of the viral genome for the insertion of DNA that is foreign to the viral genome. Such deficiencies may be in genes that are
5 essential or unessential for propagation of the adenoviral vector in tissue culture in a noncomplementing cellular host; preferably, at least one, more preferably, at least two, of the deficient genes of the inventive viral vectors are deficient for a gene that is essential
10 for propagation.

Any subtype, mixture of subtypes, or chimeric adenovirus may be used as the source of DNA for generation of the multiply deficient adenoviral vectors. However, given that the Ad5 genome has been completely
15 sequenced, the present invention will be described with respect to the Ad5 serotype.

Preferably, the adenoviral vector of the present invention is at least deficient in a function provided by one or more of the early regions, which include early
20 region 1 (E1), early region 2 (E2), including early region 2A (E2A) and/or early region 2B (E2B), early region 3 (E3), and early region 4 (E4) of the adenoviral genome. A deficiency in the E1 region is in region E1A, E1B, or both E1A and E1B. More preferably, the
25 adenoviral vector of the present invention is at least deficient in a function provided by region E1 in combination with a deficiency in region E2 (i.e., E2A, E2B, or both E2A and E2B), and/or E3, and/or E4.

Any one of the deleted regions then may be replaced
30 with a promoter-variable expression cassette to produce a foreign gene product, that is foreign with respect to adenovirus. The insertion of a foreign gene into the E2A region, for example, may be facilitated by the introduction of a unique restriction site, such that the
35 foreign gene product may be expressed from the E2A promoter.

The present invention, however, is not limited to adenoviral vectors that are deficient in gene functions only in the early region of the genome. Also included are adenoviral vectors that are deficient in the late
5 region of the genome, adenoviral vectors that are deficient in the early and late regions of the genome, as well as vectors in which essentially the entire genome has been removed, in which case it is preferred that at least either the viral inverted terminal repeats and some
10 of the promoters or the viral inverted terminal repeats and a packaging signal are left intact. One of ordinary skill in the art will appreciate that the larger the region of the adenoviral genome that is removed, the larger the piece of exogenous DNA that can be inserted
15 into the genome. For example, given that the adenoviral genome is 36 kb, by leaving the viral inverted terminal repeats and some of the promoters intact, the capacity of the adenovirus is approximately 35 kb. Alternatively, one could generate a multiply deficient adenoviral vector
20 that contains only the ITR and a packaging signal. This could then effectively allow for expression of 37-38 kb of foreign DNA from this vector.

In general, virus vector construction relies on the high level of recombination between fragments of
25 adenoviral DNA in the cell. Two or three fragments of adenoviral DNA, containing regions of similarity (or overlap) between fragments and constituting the entire length of the genome, are transfected into a cell. The host cell's recombination machinery constructs a full-
30 length viral vector genome by recombining the aforementioned fragments. Other suitable procedures for constructing viruses containing alterations in various single regions have been previously described (Berkner et al., Nucleic Acids Res., 12, 925-941 (1984); Berkner et
35 al., Nucleic Acids Res., 11, 6003-6020 (1983); Brough et al., Virology, 190, 624-634 (1992)) and can be used to

construct multiply deficient viruses; yet other suitable procedures include in vitro recombination and ligation, for example.

The first step in virus vector construction is to
5 construct a deletion or modification of a particular region of the adenoviral genome in a plasmid cassette using standard molecular biological techniques. After extensive analysis, this altered DNA (containing the deletion or modification) is then moved into a much
10 larger plasmid that contains up to one half of the adenovirus genome. The next step is to transfect the plasmid DNA (containing the deletion or modification) and a large piece of the adenovirus genome into a recipient cell. Together these two pieces of DNA encompass all of
15 the adenovirus genome plus a region of similarity. Within this region of similarity a recombination event will take place to generate a recombined viral genome that includes the deletion or modification. In the case of multiply deficient vectors, the recipient cell will
20 provide not only the recombination functions but also all missing viral functions not contained within the transfected viral genome, thus complementing any deficiencies of the recombined viral genome. The multiply deficient vector can be further modified by
25 alteration of the ITR and/or packaging signal, for example, such that the multiply deficient vector only functions or grows in a complementing cell line.

In addition, the present invention also provides complementing cell lines for propagation or growth of the
30 present inventive multiply deficient adenoviral vectors. The preferred cell lines of the present invention are characterized in complementing for at least one gene function of the gene functions comprising the E1, E2, E3 and E4 regions of the adenoviral genome. Other cell
35 lines include those that complement adenoviral vectors that are deficient in at least one gene function from the

gene functions comprising the late regions, those that complement for a combination of early and late gene functions, and those that complement for all adenoviral functions. One of ordinary skill in the art will appreciate that the cell line of choice is one that specifically complements for those functions that are missing from the recombinant multiply deficient adenoviral vector of interest and that are generated using standard molecular biological techniques. The cell lines are further characterized in containing the complementing genes in a nonoverlapping fashion, which minimizes, practically eliminating, the possibility of the vector genome recombining with the cellular DNA. Accordingly, replication-competent adenoviruses are eliminated from the vector stocks, which are, therefore, suitable for certain therapeutic purposes, especially gene therapy purposes. This also eliminates the replication of the adenoviruses in noncomplementing cells.

20 The complementing cell line must be one that is capable of expressing the products of the two or more deficient adenoviral gene functions at the appropriate level for those products in order to generate a high titer stock of recombinant adenoviral vector. For example, it is necessary to express the E2A product, DBP, at stoichiometric levels, i.e., relatively high levels, for adenoviral DNA replication, but the E2B product, Ad pol, is necessary at only catalytic levels, i.e., relatively low levels, for adenoviral DNA replication.

30 Not only must the level of the product be appropriate, the temporal expression of the product must be consistent with that seen in normal viral infection of a cell to assure a high titer stock of recombinant adenoviral vector. For example, the components necessary for viral DNA replication must be expressed before those necessary for virion assembly. In order to avoid cellular

toxicity, which often accompanies high levels of expression of the viral products, and to regulate the temporal expression of the products, inducible promoter systems are used. For example, the sheep metallothioneine

5 inducible promoter system can be used to express the complete E4 region, the open reading frame 6 of the E4 region, and the E2A region. Other examples of suitable inducible promoter systems include, but are not limited to, the bacterial lac operon, the tetracycline operon,

10 the T7 polymerase system, and combinations and chimeric constructs of eukaryotic and prokaryotic transcription factors, repressors and other components. Where the viral product to be expressed is highly toxic, it is desirable to use a bipartite inducible system, wherein

15 the inducer is carried in a viral vector and the inducible product is carried within the chromatin of the complementing cell line. Repressible/inducible expression systems, such as the tetracycline expression system and lac expression system also may be used.

20 DNA that enters a small proportion of transfected cells can become stably maintained in an even smaller fraction. Isolation of a cell line that expresses one or more transfected genes is achieved by introduction into the same cell of a second gene (marker gene) that, for

25 example, confers resistance to an antibiotic, drug or other compound. This selection is based on the fact that, in the presence of the antibiotic, drug, or other compound, the cell without the transferred gene dies, while the cell containing the transferred gene survives.

30 The surviving cells are then clonally isolated and expanded as individual cell lines. Within these cell lines are those that express both the marker gene and the gene or genes of interest. Propagation of the cells is dependent on the parental cell line and the method of

35 selection. Transfection of the cell is also dependent on cell type. The most common techniques used for

transfection are calcium phosphate precipitation, liposome, or DEAE dextran mediated DNA transfer.

Many modifications and variations of the present illustrative DNA sequences and plasmids are possible.

5 For example, the degeneracy of the genetic code allows for the substitution of nucleotides throughout polypeptide coding regions, as well as in the translational stop signal, without alteration of the encoded polypeptide coding sequence. Such substitutable
10 sequences can be deduced from the known amino acid or DNA sequence of a given gene and can be constructed by conventional synthetic or site-specific mutagenesis procedures. Synthetic DNA methods can be carried out in substantial accordance with the procedures of Itakura et
15 al., Science, 198, 1056 (1977) and Crea et al., Proc. Natl. Acad. Sci. USA, 75, 5765 (1978). Site-specific mutagenesis procedures are described in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY (2d ed. 1989). Therefore, the present
20 invention is in no way limited to the DNA sequences and plasmids specifically exemplified herein. Exemplified vectors are for gene therapy of cystic fibrosis and, therefore, contain and express the cystic fibrosis transmembrane regulator (CFTR) gene, however the vectors
25 described are easily convertible to treat other diseases including, but not limited to, other chronic lung diseases, such as emphysema, asthma, adult respiratory distress syndrome, and chronic bronchitis, as well as cancer, coronary heart disease, and other afflictions
30 suitably treated or prevented by gene therapy, vaccination, and the like. Accordingly, any gene or DNA sequence can be inserted into a multiply deficient adenoviral vector. The choice of gene or DNA sequence is one that achieves a therapeutic and/or prophylactic
35 effect, for example, in the context of gene therapy, vaccination, and the like.

One skilled in the art will appreciate that suitable methods of administering a multiply deficient adenoviral vector of the present invention to an animal for therapeutic or prophylactic purposes, e.g., gene therapy, vaccination, and the like (see, for example, Rosenfeld et al., Science, 252, 431-434 (1991), Jaffe et al., Clin. Res., 39(2), 302A (1991), Rosenfeld et al., Clin. Res., 39(2), 311A (1991), Berkner, BioTechniques, 6, 616-629 (1988)), are available, and, although more than one route can be used to administer the vector, a particular route can provide a more immediate and more effective reaction than another route. Pharmaceutically acceptable excipients are also well-known to those who are skilled in the art, and are readily available. The choice of excipient will be determined in part by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of the pharmaceutical composition of the present invention. The following formulations and methods are merely exemplary and are in no way limiting. However, oral, injectable and aerosol formulations are preferred.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the compound dissolved in diluents, such as water, saline, or orange juice; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solids or granules; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible excipients. Lozenge forms can comprise the active

ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia, emulsions, gels, and the like containing, in addition to the active ingredient, such excipients as are known in the art.

The vectors of the present invention, alone or in combination with other suitable components, can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. They also may be formulated as pharmaceuticals for non-pressured preparations, such as in a nebulizer or an atomizer.

Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid excipient, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

Additionally, the vectors employed in the present invention may be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams, or spray formulas containing, in addition to the active ingredient, such carriers as are known in the art to be appropriate.

The dose administered to an animal, particularly a human, in the context of the present invention will vary with the gene or other sequence of interest, the composition employed, the method of administration, and the particular site and organism being treated. The dose should be sufficient to effect a desirable response, e.g., therapeutic or prophylactic response, within a desirable time frame.

The multiply deficient adenoviral vectors and complementing cell lines of the present invention also have utility in vitro. For example, they can be used to study adenoviral gene function and assembly, or expression of foreign DNA in a suitable target cell. One of ordinary skill can identify a suitable target cell by selecting one that can be transfected by the inventive adenoviral vector and/or infected by adenoviral particles, resulting in expression of the thereby inserted adenoviral DNA complement. Preferably, a suitable target cell is selected that has receptors for attachment and penetration of adenovirus into a cell. Such cells include, but are not limited to, those originally isolated from any mammal. Once the suitable target cell has been selected, the target cell is contacted with a foreign DNA-containing recombinant multiply deficient adenoviral vector or adenoviral particle of the present invention, thereby effecting transfection or infection, respectively. Expression, toxicity, and other parameters relating to the insertion and activity of the foreign DNA in the target cell is then measured using conventional methods well known in the art. In so doing, researchers can learn and

cells of the individual's body. Such a matrix may be any suitable biocompatible material, including collagen, cellulose, and the like. Of course, alternatively or in addition, once having observed a positive response to the
5 in vitro test, the transfection can be implemented in situ by administration means as detailed hereinabove.

The following examples further illustrate the present invention and, of course, should not be construed as in any way limiting its scope. Enzymes referred to in
10 the examples are available, unless otherwise indicated, from Bethesda Research Laboratories (BRL), Gaithersburg, MD 20877, New England Biolabs Inc. (NEB), Beverly, MA 01915, or Boehringer Mannheim Biochemicals (BMB), 7941 Castleway Drive, Indianapolis, IN 46250, and are used in
15 substantial accordance with the manufacturer's recommendations. Many of the techniques employed herein are well known to those in the art. Molecular biology techniques are described in detail in suitable laboratory manuals, such as Maniatis et al., Molecular Cloning: A
20 Laboratory Manual, Cold Spring Harbor, NY (2d ed. 1989), and Current Protocols in Molecular Biology (Ausubel et al., eds. (1987)). One of ordinary skill in the art will recognize that alternate procedures can be substituted for various procedures presented below. Although the
25 examples and figures relate to Ad_{Gv}.10, Ad_{Gv}.11, Ad_{Gv}.12, and Ad_{Gv}.13 which contain the cystic fibrosis transmembrane regulator gene (CFTR), namely Ad_{Gv}CFTR.10, Ad_{Gv}CFTR.11, Ad_{Gv}CFTR.12, and Ad_{Gv}CFTR.13, these vectors are not limited to expression of the CFTR gene and can be
30 used to express other genes and DNA sequences. For example, therefore, the present invention encompasses such vectors comprising any suitable DNA sequence, which may be a foreign gene, a fragment thereof, or any other DNA sequence. Such a suitable DNA sequence may have use
35 in gene therapy to treat a disease that is suitably treated by gene therapy. Alternatively, a suitable DNA

sequence may also have a prophylactic use, such as when the DNA sequence is capable of being expressed in a mammal resulting in, for example, a polypeptide capable of eliciting an immune response to the polypeptide, as used in vaccination. Yet another alternative use of a suitable DNA sequence capable of being expressed in a mammal is to provide any other suitable therapeutic and/or prophylactic agent, such as an antisense molecule, particularly an antisense molecule selected from the group consisting of MRNA and a synthetic oligonucleotide.

Example 1

This example describes the generation of one embodiment involving Ad_{Gv}.10, namely Ad_{Gv}CFTR.10, which is deficient in the E1 and E3 regions.

Ad_{Gv}CFTR.10 expresses the CFTR gene from the cytomegalovirus (CMV) early promoter. Two generations of this vector have been constructed and are designated Ad_{Gv}CFTR.10L and Ad_{Gv}CFTR.10R, dependent on the direction in which the CFTR expression cassette is placed in the E1 region in relation to the vector genome as shown in Figure 1, which is a set of schematic diagrams of Ad_{Gv}CFTR.10L and Ad_{Gv}CFTR.10R.

The CFTR expression cassette was constructed as follows. pRK5 (Genentech Inc., South San Francisco, CA) was digested with Kpn I (New England Biolabs (NEB), Beverly, MA), blunt-ended with Mung Bean nuclease (NEB), and an Xho I linker (NEB) was ligated in place of the Kpn I site. The resulting vector was named pRK5-Xho I. pRK5-Xho I was then digested with Sma I (NEB) and Hin dIII (NEB) and blunt-ended with Mung bean nuclease. A plasmid containing the CFTR gene, pBQ4.7 (Dr. Lap-Chee Tsui, Hospital for Sick Children, Toronto, Canada), was digested with Ava I (NEB) and Sac I (NEB) and blunt-ended with Mung bean nuclease. These two fragments were

isolated and ligated together to produce pRK5-CFTR1, the CFTR expression cassette.

pRK5-CFTR1 was digested with Spe I (NEB) and Xho I and blunt-ended with Klenow (NEB). pAd60.454 (Dr. L.E. Babiss, The Rockefeller University, New York, NY), which contains Ad5 sequences from 1-454/3325-5788, was digested with Bgl II (NEB) and blunt-ended with Klenow. These two fragments were purified from vector sequences by low-melt agarose technique (Maniatis et al., Molecular Cloning: a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (2nd ed. 1989)) and ligated together to produce the left arm plasmids pGVCFTR.10L and pGVCFTR.10R.

The left arm plasmid from pGVCFTR.10L or pGVCFTR.10R was digested with Nhe I (NEB). The right arm of the virus was produced by digesting Ad5d1324 (Dr. Thomas E. Shenk, Princeton University, Princeton, NJ) with Cla I (NEB). The two fragments, a small 918 bp fragment and a large approximately 32,800 bp fragment, were separated by sucrose gradient centrifugation (Maniatis et al., supra). The large fragment was mixed with the left arm plasmid fragments and transfected into 293 cells by standard calcium phosphate protocol (Graham et al., Virology, 52, 456 (1973)). The resulting recombinant viruses were plaque-purified on 293 cells, and viral stocks were established using standard virology techniques (e.g., Berkner et al., (1983) and (1984), supra).

Example 2

This example describes the generation of Ad_{GV}CFTR.11, which is deficient in the E1, E3, and E4 regions.

Ad_{GV}CFTR.11 was constructed by means of a single in vivo recombination between 1-27082, i.e., the left arm, of Ad_{GV}CFTR.10 and a plasmid (pGV11A, pGV11B, pGV11C, or pGV11D; described in detail below) containing 21562-35935, i.e., the right arm, of Ad5 linearized with Bam HI

(NEB) and Sal I (NEB) and into which the various E3 and E4 alterations as described below were introduced.

The left arm from Ad_{Gv} CFTR.10 was isolated on a concave 10-40% sucrose gradient, wherein 1/4th of the total solution was 40%, after intact Ad_{Gv}CFTR.10 was
5 digested with Spe I (NEB) and Srf I (Stratagene, La Jolla, CA) to yield the 1-27082 bp fragment.

The right arm was obtained by Bam HI-Sal I digestion of a modified pGEM vector (pGBS). pGBS was generated as
10 follows. pGemI (Promega, Madison, WI) was digested with Eco RI and blunt-ended with Klenow, and a Sal I linker was ligated into the vector. The resulting DNA was then digested with Sal I and religated, thereby replacing the Eco RI site with a Sal I site and deleting the sequence
15 between the two Sal I sites, to generate pGEMH/P/S, which was digested with Hin DIII and blunt-ended with Klenow, and a Bam HI linker was ligated into the vector to generate pGEMS/B. pGEMS/B was digested with Bam HI and Sal I and ligated with an ~ 14 kb Bam HI-Sal I fragment
20 (21562-35935 from Ad5) from a pBR plasmid called p50-100 (Dr. Paul Freimuth, Columbia University, NY) to generate pGBS.

Three different versions of the right arm plasmid were constructed in order to introduce into the
25 adenoviral vector two Ad E3 gene products having anti-immunity and anti-inflammatory properties. The large E3 deletion in pGBSΔE3ORF6, designated pGV11(0) (Example 7), was essentially replaced with three different versions of an expression cassette containing the Rous sarcoma virus-
30 long terminal repeat (RSV-LTR) promoter driving expression of a bicistronic mRNA containing at the 5' end the Ad2 E3 19 kDa anti-immunity gene product and at the 3' end the Ad5 E3 14.7 Kda anti-inflammatory gene product. One additional virus was constructed by
35 deleting the 19 kDa cDNA fragment by Bst BI (NEB) fragment deletion. This virus, designated Ad_{Gv}CFTR.11(D),

contains the RSV-LTR promoter driving expression of a monocistronic mRNA containing only the E3 14.7 kDa anti-inflammatory gene product.

The Spe I (27082) - Nde I (31089) fragment from pGBSΔE3 (Example 5) was subcloned into pUC 19 by first cloning the Eco RI (27331) - Nde I (31089) fragment into identical sites in the PUC 19 polylinker. A Hin dIII (26328) - Eco RI (27331) fragment generated from pGBS was then cloned into the Eco RI site of this clone to generate pHNΔE3. Using appropriate primers, a PCR fragment with flanking Xba I sites was generated containing the RSV-LTR promoter, the Ad2 E3 19 kDa gene product, and the Ad5 E3 14.7 kDa gene product. The amplified fragment was digested with Xba I and subcloned into pUC 19 to generate pXA. After analysis of the Xba I fragment, the fragment was ligated into pHNΔE3 to generate pHNRA.

Using appropriate primers, two PCR fragments with flanking Bst BI sites were generated that encode internal ribosomal entry sites (IRES), which are known to enhance the translation of mRNAs that contain them (Jobling et al., Nature, 325, 622-625 (1987); Jang et al., Genes and Development, 4, 1560-1572 (1990)). One fragment (version B) contains a 34 bp IRES from the untranslated leader of the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4 leader) (Jobling et al., supra). The other fragment (version C) contains a 570 bp IRES from the 5' nontranslated region of encephalomyocarditis virus (EMCV) mRNA (Jang et al., supra). Each Bst BI fragment from version B or C was cloned in place of the Bst BI fragment in pXA. The resulting plasmids, named pXB and pXC, respectively, were moved into pHNΔE3 to generate pHNRB and pHNRC, respectively, after sequence analysis of the Xba I fragments.

The Spe I (27082) - Nde I (31089) fragment from pGBSΔE3ORF6 was replaced with the Spe I - Nde I fragments

from pHNRA, pHNRB, pHNRC and pHNRD to generate pGV11A, pGV11B, pGV11C and pGV11D, respectively.

The pGBV plasmid DNA was linearized with Bam HI and Sal I and mixed with the purified left arm DNA fragment
5 in varying concentrations to give about 20 μ g total DNA, using salmon sperm or calf thymus DNA (Life Technologies, Gaithersburg, MA) to bring the amount of DNA to about 20 μ g as needed. The mixed fragments were then transfected into 293 cells using standard calcium phosphate
10 techniques (Graham et al., supra).

Five days after transfection, the cell monolayer was harvested by freeze-thawing three times. The resulting hybrid virus was titered onto 293 cells and isolated plaques were picked. The process of plaque isolation was
15 repeated twice more to ensure a single recombinant virus existed in the initial plaque stock. The plaque isolate stock was then amplified to a large viral stock according to standard virology techniques as described in Burlleson et al., Virology: a Laboratory Manual, Academic Press
20 Inc. (1992).

Figure 2 is a set of schematic diagrams of the various AD_{Gv}CFTR.11 viral vectors. The diagrams are aligned with that of AD_{Gv}CFTR.10L for comparison.

Example 3

25 This example describes the generation of Ad_{Gv}CFTR.12, which is deficient in the E1, E3, and E4 regions.

Ad_{Gv}.12 is characterized by complete elimination of the E4 region. This large deletion allows for insertion of up to about 10 kb of exogenous DNA. More importantly,
30 another region of the genome is accessible for introduction of foreign DNA expression cassettes using the Ad_{Gv}CFTR.12 vectors. This deletion now enables the incorporation of larger expression cassettes for other products. For example, soluble receptors, i.e., TNF or
35 IL-6 without a transmembrane domain so that they are now

not attached to the membrane, and antisense molecules, e.g., those directed against cell cycle regulating products, such as cdc2, cdk kinases, cyclins, i.e., cyclin E or cyclin D, and transcription factors, i.e., E2F or c-myc, to eliminate inflammation and immune responses.

pGV11(0) is altered to produce a right arm plasmid in which the entire E4 region is deleted. The resulting plasmid in which the entire E3 and E4 regions are deleted is named pGV12(0). This is done by introducing a Pac I restriction site at the Afl III site at 32811 and the Bsg I site at 35640. Deletion of the Pac I fragment between these two sites effectively eliminates all of the E4 sequences including the E4 TATA element within the E4 promoter and the E4 poly A site.

Virus construction is performed as previously described except that the 293/E4 cell line or the 293/ORF6 cell line is used. The left arm from Ad_{Gv}CFTR.10L, the right arm pGV12(0) plasmid, and all other general techniques are as described in Example 2. Since E4 contains essential gene products necessary for viral growth, the resulting E4 deletion mutant virus cannot grow in the absence of exogenously expressed E4. Therefore, all manipulations for viral construction are carried out in the new 293/E4 cell line or 293/ORF6 cell line (described in Examples 8 and 9, respectively). The resulting virus is Ad_{Gv}CFTR.12, which is represented schematically in Figure 3, alone with Ad_{Gv}CFTR.10L for comparison.

Example 4

This example describes the generation of Ad_{Gv}CFTR.13, which is deficient in the E1, E2A, E3, and E4 regions.

Ad_{Gv}.13 is characterized by not only complete elimination of E1 and E4 (as in AD_{Gv}.12) but also complete elimination of E2A. The complete coding region of E2A is

deleted by fusing together the DNA from two E2A mutant viruses, namely H5in800 and H5in804, containing insertions of Cla I restriction sites at both ends of the open reading frame (Vos et al., Virology, 172, 634-642 (1989); Brough et al., Virology, 190, 624-634 (1992)). The Cla I site of H5in800 is between codons 2 and 3 of the gene, and the Cla I site of H5in804 is within the stop codon of the E2A gene. The resultant virus contains an open reading frame consisting of 23 amino acids that have no similarity to the E2A reading frame. More importantly, this cassette offers yet another region of the virus genome into which a unique gene can be introduced. This can be done by inserting the gene of interest into the proper reading frame of the existing mini-ORF or by introducing yet another expression cassette containing its own promoter sequences, polyadenylation signals, and stop sequences in addition to the gene of interest.

Adenovirus DNA is prepared from H5in800 and H5in804. After digestion with the restriction enzyme Hin dIII (NEB), the Hin dIII A fragments from both H5in800 and H5in804 are cloned into pKS+ (Stratagene). The resulting plasmids are named pKS+H5in800Hin dIIIA and pKS+H5in804Hin dIIIA, respectively. The Cla I (NEB) fragment from pKS+H5in800Hin dIIIA is then isolated and cloned in place of the identical Cla I fragment from pKS+H5in804Hin dIIIA. This chimeric plasmid, pHin dIIIA Δ E2A effectively removes all of the E2A reading frame as described above. At this point, the E2A deletion is moved at Bam HI (NEB) and Spe I (NEB) restriction sites to replace the wild-type sequences in pGV12(0) to construct pGV13(0).

Ad_{GV}CFTR.13 virus (see Figure 4) is constructed as previously described by using Ad_{GV}CFTR.10 left arm DNA and pGV13(0) right arm plasmid DNA. However, the recipient cell line for this virus construction is the triple

This example describes the generation of pGBSΔE3.
This plasmid was generated to remove the majority of the E3 region within pGBS, including the E3 promoter and existing E3 genes, to make room for other constructs and to facilitate introduction of E3 expression cassettes. This plasmid contains a deletion from 28331 to 30469.

Example 6

A Pac I site was generated in place of the Mun I site at 32830 by treating pGBS Mun I-digested DNA with Klenow to blunt-end the fragment and by ligating a Pac I linker to this. The modified DNA was then digested with Nde I and the resulting 1736 bp fragment (Nde I 31089 - Pac I 32830) was gel-purified. A PCR fragment was prepared using A5 (35564)P (IDT, Coralville, IA) and T7 primers (IDT, Coralville, IA) and pGBS as template. The resulting fragment was digested with Pac I and Sal I to generate Pac I 35566 - Sal I 35935. A Sma I site within the polylinker region of pUC 19 was modified to a Pac I

site by ligating in a Pac I linker. The Pac I 35566 - Sal I 35935 fragment was moved into the modified pUC 19 vector at Pac I and Sal I sites, respectively, in the polylinker region. The modified Nde I 31089 - Pac I 32830 fragment was moved into the pUC 19 plasmid, into which the Pac I 35566 - Sal I 35935 fragment already had been inserted, at Nde I and Pac I sites, respectively. The Nde I 31089 - Sal I 35935 fragment from the pUC 19 plasmid was purified by gel purification and cloned in place of the respective Nde I and Sal I sites in pGBSΔE3 to yield pGBSΔE3ΔE4.

Example 7

This example describes the generation of pGBSΔE3ORF6.

15 The Ad5 894 bp E4 ORF-6 gene was placed 3' of the E4 promoter in pGBSΔE3ΔE4. ORF-6 is the only absolutely essential E4 product necessary for virus growth in a non-E4 complementing cell line. Therefore, this product was re-introduced into the right arm plasmid (Example 2) under its own promoter control so that Ad_{Gv}CFTR.11 virus can be propagated in 293 cells.

A PCR fragment was generated using A5s(33190)P (32 bp; 5'CACTTAATTAACGCCTACATGGGGGTAGAGT3') (SEQ ID NO:1) and A5a(34084)P (34 bp; 5'CACTTAATTAAGGAAATATGACTACGTCCGGCGT3') (SEQ ID NO:2) as primers (IDT, Coralville, IA) and pGBS as template. This fragment was digested with Pac I and gel-purified. The product was introduced into the single Pac I site in pGBSΔE3ΔE4 to generate pGV11(0), which was the plasmid that was E3-modified for expression of the 19 kDa and 14.7 kDa Ad E3 products.

Example 8

This example describes the generation of the 293/E4 cell line.

The vector pSMT/E4 was generated as follows. A 2752 bp Mun I (site 32825 of Ad2) - Sph I (polylinker) fragment was isolated from pE4(89-99), which is a pUC19 plasmid into which was subcloned region 32264-35577 from Ad2, blunt-ended with Klenow, and treated with phosphatase (NEB). The 2752 bp Mun I-Sph I fragment was then ligated into pMT010/A⁺ (McNeall et al., Gene, 76, 81-89 (1989)), which had been linearized with Bam HI, blunt-ended with Klenow and treated with phosphatase, to generate the expression cassette plasmid, pSMT/E4.

The cell line 293 (ATCC CRL 1573; American Type Culture Collection, Rockville, MD) was cultured in 10% fetal bovine serum Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MA). The 293 cells were then transfected with pSMT/E4 linearized with Eco RI by the calcium phosphate method (Sambrook et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)). Approximately 24-48 hours post-transfection, medium (as above) containing 100 μ M methotrexate and amethopterin (Sigma Chemical Co., St. Louis, MO) was added. The presence of methotrexate in the medium selects for expression of the dihydrofolate reductase (DHFR) gene, which is the selectable marker on the pSMT/E4 plasmid.

The normal cell DHFR gene is inhibited by a given concentration of methotrexate (cell type-specific), causing cell death. The expression of the additional DHFR gene in transfected cells containing pSMT/E4 provides resistance to methotrexate. Therefore, transfected cells containing the new genes are the only ones that grow under these conditions (for review, see Sambrook et al., supra).

When small colonies of cells formed from the initial single cell having the selectable marker, they were clonally isolated and propagated (for review, see Sambrook et al., supra). These clones were expanded to

produce cell lines that were screened for expression of the product -- in this case, E4 -- and screened for functionality in complementing defective viruses -- in this case, both E1 and E4 defective viruses.

- 5 The result of this process produced the first 293/E4 cell lines capable of complementing adenoviral vectors defective in both E1 and E4 functions, such as Ad_{Gv}CFTR.12.

Example 9

- 10 This example describes the generation of the 293/ORF-6 cell line.

 The primers A5s(33190)P and A5a(34084)P were used in a polymerase chain reaction (PCR) (PCR Protocols, A guide to Methods and Applications, Innis et al., eds., Academic Press, Inc. (1990)) to amplify the ORF-6 gene of Ad5 E4 and generate Pac I sites at the ends for cloning. The amplified fragment was blunt-ended with Klenow and cloned into pCR-Script SK(+) (Stratagene, La Jolla, CA). The resulting plasmid, pCR/ORF-6, was sequenced and then the

15 ORF-6 insert was transferred into the pSMT/puro expression vector, which was generated by ligation of a blunt-ended Eco RI - Hin dIII fragment containing the SMT promoter into the blunt-ended Mlu I-Hin dIII site in pRCpuro, to generate pSMT/ORF-6.

- 20 The 293 cell line was cultured and transfected with pSMT/ORF-6 as described in Example 8, except that the transfected cells were placed under selection for the puromycin resistance gene, which allows cells that express it to grow in the presence of puromycin.
- 25 Colonies of transformed cells were subcloned and propagated and were screened as described in Example 8.

 This cell line is suitable for complementing vectors that are deficient in the E1 and E4 regions, such as the Ad_{Gv}CFTR.12 series of vectors.

Example 10

This example describes the generation of the 293/E4/E2A cell line. The 293/E4/E2A cell line allows E1, E4, and E2A defective viral vectors to grow.

- 5 The E2A expression cassette (see Figure 5) for introduction into 293/E4 cells is produced as follows. The first step is to alter surrounding bases of the ATG of E2A to make a perfect Kozak consensus (Kozak, J. Molec. Biol., 196, 947-950 (1987)) to optimize expression
- 10 of E2A. Two primers are designed to alter the 5' region of the E2A gene. Ad5s(23884), an 18 bp oligonucleotide (5'gCCgCCTCATCCgCTTT3') (SEQ ID NO:3), is designed to prime the internal region flanking the Sma I site of the E2A gene. DBP(ATG)R1, a 32 bp oligonucleotide
- 15 (5'CCggAATTCACCATggCgAgtcgggAAGAgg3') (SEQ ID NO:4), is designed to introduce the translational consensus sequence around the ATG of the E2A gene modifying it into a perfect Kozak extended consensus sequence and to introduce an Eco RI site just 5' to facilitate cloning.
- 20 The resulting PCR product using the above primers is digested with Eco RI and Sma I (NEB) and cloned into the identical polylinker sites of pBluescript IIKS+ (Stratagene, La Jolla, CA). The resulting plasmid is named pKS/ESDBP.
- 25 A Sma I-Xba I fragment is isolated from pHRKauffman (Morin et al., Mol. Cell. Biol., 9, 4372-4380 (1989)) and cloned into the corresponding Sma I and Xba I sites of pKS/ESDBP to complete the E2A reading frame. The resulting plasmid is named pKSDBP. In order to eliminate
- 30 all homologous sequences from vector contained within the expression cassette, the Kpn I to Dra I fragment from pKSDBP is moved into corresponding Kpn I and Pme I sites in PNEB193 (NEB) in which the Eco RI sites in the polylinker have been destroyed (GenVec, Rockville, MD).
- 35 The resulting clone, pE2A, contains all of the E2A

reading frame without any extra sequences homologous to the E2A deleted vector in Example 4.

A 5' splice cassette is then moved into pE2A to allow proper nuclear processing of the mRNA and to
5 enhance expression of E2A further. To do this, pRK5, described in Example 1, is digested with Sac II (NEB), blunt-ended with Mung Bean nuclease (NEB), and digested with Eco RI (NEB). The resulting approx. 240 bp fragment of interest containing the splicing signals is cloned
10 into the Cla I (blunt-ended with Klenow) to Eco RI sites of pE2A to generate p5'E2A. The blunt-ended (Klenow) Sal I to Hin dIII fragment from p5'E2A containing the E2A sequences is moved into the blunt-ended (Klenow) Xba I site of pSMT/puro and pSMT/neo. The resulting E2A is
15 named pKSE2A.

The Xba I fragment from pKSE2A that contained all the E2A gene is moved into the Xba I site of pSMT/puro and pSMT/neo. The resulting E2A expression plasmids, pSMT/E2A/puro and pSMT/E2A/neo, are transfected into
20 293/E4 and 203/ORF-6 cells, respectively. Cells transfected with pSMT/E2A/puro are selected for growth in standard media plus puromycin, and cells transfected with pSMT/E2A/neo are selected for growth in standard media plus Geneticin (G418; Life Technologies, Gaithersburg,
25 MD). Clonal expansion of isolated colonies is as described in Example 8. The resulting cell lines are screened for their ability to complement E1, E4, and E2A defective viral vectors.

These cell lines are suitable for complementing
30 vectors that are deficient in the E1, E4, and E2A regions of the virus, such as those described in the Ad_{gv}CFTR.13 series of viral vectors.

Example 11

This example describes the generation of complementing cell lines using the cell line A549 (ATCC) as the parental line.

- 5 Ad2 virus DNA is prepared by techniques previously described. The genomic DNA is digested with Ssp I and Xho I, and the 5438 bp fragment is purified and cloned into Eco RV/Xho I sites of pKS+ (Stratagene) to produce pKS341-5778. After diagnostic determination of the
- 10 clone, an Xho I (blunt-ended with Klenow) to Eco RI fragment is moved into Nru I (blunt) to Eco RI sites in pRC/CMVneo to produce pE1neo. Transformation of A549 cells with this clone yields a complementing cell line (similar to 293), wherein additional expression cassettes
- 15 can be introduced, in a manner similar to that described for the 293 cell, to produce multicomplementing cell lines with excellent plaqueing potential.

Example 12

- This example sets forth a protocol for the
- 20 generation of 293/E2A cell lines and use thereof to construct an adenoviral vector that is defective in both E1 and E2A regions.

- An E2A expression cassette vector was obtained, as described in Example 10 and depicted in Figure 5. The
- 25 E2A expression cassette vector includes the gene that confers neomycin resistance as a marker for transfected cells.

- Also as described in Example 10, 293 cells were transfected with pSMT/E2A/neo and the transfected cells
- 30 were selected for growth in standard media plus G418. Clonal expansion of the selected cells was effected as described in Example 8. The resulting cell lines were screened for their ability to express the DNA-binding protein (DBP; the product of the E2A gene) upon induction

and their ability to complement E1 and E2A defective viral vectors.

For testing the ability of the neomycin positive (neo⁺; i.e., resistant to neomycin) clonal isolates of the 293/E2A cell lines for their ability to express DBP, cells were grown in the presence of G418 to maintain selection. Established monolayers from independent clonal isolates were induced with 100 μ M ZnCl₂ for 24 hours and the expression of the DBP gene was detected by immunoblotting, using a standard method. Of the 62 lines that were tested, 42% of the neo⁺ cell lines were positive for DBP expression (DBP⁺), and all of the DBP⁺ cell lines showed inducible DBP expression. The following Table 1 presents the data from the DBP expression screen:

Table 1

	Cell line	DBP expression	Cell line	DBP expression
	3	-	202	-
	6	-	203	-
20	9	-	207	-
	10	+	208	-
	12	+	210	-
	13	+	211	-
	16	-	212	+
25	17	+	213	-
	19	+	215	-
	21	+	216	+
	32	+	219	-
	35	+	301	+
30	36	-	302	-
	39	+	305	-
	41	-	307	-
	42	-	309	-
	43	-	311	-
35	52	-	313	-

	Cell line	DBP expression	Cell line	DBP expression
	54	+	314	-
	55	-	315	-
	57	+	316	-
	58	+	317	-
5	60	-	321	+
	61	+	323	-
	62	-	324	-
	104	+	325	+
	107	-	326	-
10	108	+	327	+
	111	-	328	+
	112	+	329	+
	201	+	330	+

The clonal 293/E2A cell lines were subsequently
 15 screened for the level of induced DBP expression via
 immunoblotting, using the method of Brough et al., supra,
 the results of which are depicted in the autoradiograms
 labelled as Figures 6 and 7. Those cell lines that
 accumulated a similar level of DBP as that in the HeLa-
 20 based gmDBP2 cells were further analyzed. As noted in
 Figure 6, based on the lysates of induced cells, the
 level of induced DBP expression varied widely in the
 clonal isolates. For example, cell lines 104, 112, and
 216 produced a substantial amount of DBP upon induction
 25 as described above, whereas cell lines 19 and 61 produced
 no more than that produced by gmDBP2 cells.

The clonal 293/E2A cell lines were also analyzed for
 their ability to accumulate DBP over the first 24 hours
 of induction, again using the method of Brough et al.,
 30 supra. As noted in Figure 7, based on lysates of cells
 harvested at 0, 2, 16, and 24 hours post-induction,
 several lines were noted to progressively accumulate DBP
 over the incubation period, consistent with virus growth.

For testing complementation by the resulting 293/E2A cell lines, E2A deletion virus was tested for growth on these cell lines using conventional techniques. As is well known in the art, viral growth can be measured semiquantitatively by simple observation of plaque formation in a monolayer of host cells, which was done here. The same lines were tested for their relative expression of the E2A gene, i.e., the relative expression of DBP was measured via immunoblot in accordance with Brough et al., *Virology*, 190, 624-634 (1992). The relative level of expression or growth with respect to the aforementioned parameters (lowest +/- to highest +++) of each of the cell lines tested is set forth in Table 2:

15

Table 2

	Cell Line	Relative level of DBP Expression	Ability to support an E2A deletion virus for plaque formation
	54	+++++	++++
	61	++	+
	104	+++++	-
20	112	++++	+++++
	201	++++	++
	208	-	-
	212	+++	+
	216	++++	+/-
25	325	+	-
	327	+++	-
	328	+++	-
	330	+++++	-

As reflected in Table 2, the result of this study showed that two 293/E2A cell lines (namely 54 and 112) support E2A deletion virus plaque formation and, thus, growth.

30

The selected cell lines were also shown to complement vectors that are deficient in the E1 and the E2A regions of the virus, using cell culturing methods that are routine to the art. Such a doubly deficient vector was generated using methods disclosed in Examples 1 and 3. Figure 8 displays the structure of Ad_{Gv}CFTR.12B, which is an adenoviral vector deficient for E1 and E2A regions. Presence of the Ad_{Gv}CFTR.12B vector in three different lysates of transfected cells after passaging of the cells was noted by detecting the sequences of DNA associated with the vector via a standard PCR assay. The three different lysates were tested separately for the presence of CFTR sequences (columns labelled "A" in Figure 9), absence of E2A sequences, i.e., evidence of the deletion (columns labelled "B"), and presence of wildtype E2A sequences (columns labelled "C"). The experiment can be analyzed from the reverse contrast photograph of the ethidium bromide-stained, gel-separated fragments of DNA depicted in Figure 9, which was accomplished using standard methods.

The results show that all three lysates contain CFTR and E2A deletion sequences, which is consistent with the structure of the Ad_{Gv}CFTR.12B vector. No wildtype E2A sequences could be detected in these lysates. In Figure 9, "M" signifies a DNA marker to verify product size, "+" denotes a sample in which the positive template for the given primer set was used, "-" denotes a negative primer used for each given primer set, and, as noted above, A, B, and C stand for the three viral lysates tested.

Accordingly, an adenoviral vector having deletions at the E1 and E2A regions has been generated, and cell lines having the ability to complement the doubly deficient vector have been identified.

Example 13

This example illustrates the use of an E2A deletion plasmid for the expression of a foreign DNA.

The E2A deletion plasmid pGV13(0), as described in Example 4, was used to construct a GV12B series of vectors. Modifications of the pGV13(0) included substituting a unique *SceI* restriction site for the *ClaI* site and changing the region surrounding the ATG of the E2A gene to an optimized Kozak consensus sequence. A marker gene (β -glucuronidase) having flanking *SceI* restriction sites was inserted in place of the E2A gene such that the marker gene expresses in response to all of the signals used to express the most abundant early gene, i.e., DBP. The resulting plasmid (pGBSE2GUS) was tested for functionality by transfection and subsequent assessment of β -glucuronidase activity; all transfected cell lines tested showed high levels of expression of β -glucuronidase, which is detected by the generation of a blue color when β -glucuronidase catalyzes a reaction with the substrate X-glu.

Another viral vector (Ad_{GV}Luc;E2GUS), which is depicted in Figure 10, was constructed to demonstrate the utility of the deleted E2 region for placement of a foreign DNA for gene therapy purposes, for example. The Ad_{GV}Luc;E2GUS vector contains the CMV luciferase marker in the E1 region and the E2 β -glucuronidase in the E2A region. The predecessor vector (Ad_{GV}LUC.10) was used to transfect 293/E2A cells; a subsequent staining of the resulting viral plaques for β -glucuronidase using X-glu revealed virtually no blue color, i.e., no β -glucuronidase activity was detected. Plaques that formed from 293/E2A cells transfected with the Ad_{GV}Luc;E2GUS vector generated a substantial amount of blue color upon the addition of X-gal.

Accordingly, a foreign DNA substituted at the E2A region of an adenoviral vector can function.

Example 14

This example sets forth a protocol for the generation of 293/ORF6 cell lines and use thereof to construct an adenoviral vector that is defective in both E1 and E4 regions.

The E4-ORF6 expression cassette depicted in Figure 11 was constructed using methods disclosed in Example 9 above. Transfection of 293 cells was effected with pSMT/ORF6/puro, and transfected cells were selected for growth in standard media plus puromycin. Clonal expansion was effected as described in Example 8. The resulting cell lines were screened for their ability to express E4-ORF6 upon induction and their ability to complement E1 and E4 defective viral vectors.

Puromycin positive (puro⁺; i.e., puromycin resistant) clonal isolates of the 293/ORF6 cell lines were screened for their ability to express ORF6. Cells were grown in the presence of puromycin to maintain selection. Established monolayers from independent clonal isolates were induced with 100 μ M ZnCl₂ for 24 hours. The expression of the ORF6 gene was detected by Northern blotting, thereby identifying the RNA transcript. The relative level of expression (lowest (+) to highest +++) of each of the cell lines tested is set forth in Table 3:

Table 3

	Cell line	ORF6 expression	Cell line	ORF6 expression
	A2	+++	B8	++
	A2-1	(+)	B8-1	(+)
30	A2-2	(+)	B8-2	+++
	A2-3	-	B8-3	+
	A2-4	(+)	B8-4	+
	A2-5	(+)	B8-5	(+)
	A2-6	-	B8-6	-
35	A2-7	(+)	B8-7	+
	A2-8	-	B8-8	++

	Cell line	ORF6 expression	Cell line	ORF6 expression
	A2-9	(+)	B8-9	++
	A2-10	-	B8-10	(+)
	A2-11	-	B8-14	(+)
	A2-12	+	B8-16	-
5	A2-13	-	B8-18	-
	A2-14	+++++	B8-19	-
	A2-24	-	B8-20	-
	A2-59	-	B8-21	-
	B8-22	-	B8-23	-
10	B8-24	-	B8-27	-

The result of the test for expression of the ORF6 gene was that 53% of the puromycin-resistant cell lines were positive for ORF6 transcripts, and all such positive ORF6 cell lines were shown to be inducible for ORF6 expression.

PCR was also used to detect insertion of a gene at the E4 deletion region of an adenoviral vector, the results of which are depicted in Figure 12. Passaged lysates of Ad_{Gv}βgal.12 transfected cells were subjected to PCR that amplified certain gene sequences associated with wildtype E4, namely 3087 bp and 367 bp fragments. DNA from the lysates of mock infected 293/ORF6 cells (lane 1), Ad_{Gv}βgal.10 infected cells (lane 2), and Ad_{Gv}βgal.12 infected cells (lane 3) were subjected to gel electrophoresis and ethidium bromide staining. The reverse contrast photograph provided in Figure 12, which is of the resulting stained gel, indicates that the Ad_{Gv}βgal.10 vector is missing the portion of the E4 region that includes the 367 bp sequence and the Ad_{Gv}βgal.12 vector is missing the portion of the E4 region that includes the 3087 bp sequence.

Growth of an E4 deleted vector was monitored in the 293/ORF6 cell lines. Cells were infected at a multiplicity of infection (moi) of 10, and the amount of

virus growth was monitored by complementary plaque analysis after 5 days of growth. The results are depicted in Figure 13, which is a bar graph that indicates the plaque forming units (PFU) per cell on the y-axis and identifies the cell line tested on the x-axis. For the positive control growth, the cell line W162 was used, which is a cell line that is known to complement E4 function. For the negative control, the 293 cell line was used, which is known to complement only for E1 function. Cell lines A2, B8, 216, and 406 are independent isolates of 293/ORF6 cell lines that show varying quantitative complementation of the E4 deletion virus (dl366).

Accordingly, cell lines have been identified that complement E4 function, thereby allowing growth of E4 deletion virus, which has been shown to be capable of harboring functioning foreign DNAs. These cell lines are suitable for complementing vectors that are doubly deficient for E1 and E4 regions of the virus, such as those described in the Ad_{gy}CFTR.12 series above or as shown in Figure 14, which is a schematic representation of the Ad_{gy} β -gal.12 vector. The Ad_{gy} β -gal.12 vector has the β -galactosidase gene inserted at the E1 region and a deleted E4 region.

Example 15

This example illustrates uses of adenoviral vectors having E1 and E4 deletions.

The E4 deletion plasmid, pGBS Δ E4, has been modified to contain several unique restriction sites. These sites are used to clone any suitable foreign DNA into this region, using the adenoviral E4 promoter for expression. As noted above, cloning β -glucuronidase at this region resulted in a perfectly functional and foreign DNA expressing viral vector. Accordingly, a suitable foreign DNA placed at the E1 region and another suitable foreign

DNA at the E4 region, both on the same viral vector, can express the respective foreign DNAs using the control of the E1 and E4 promoters or other promoters as desired.

A second modification of the E4 region allows for
5 expression of a suitable foreign DNA from a variety of heterologous control elements. The plasmid construct was built in such a way so that multiple exchanges can be made conveniently. The multiplasmid contains the following features that can be exchanged conveniently:

- 10 1. an adenoviral segment used for homologous recombination, ligation to place the foreign DNA at either the E1 end or E4 end of the vector;
2. a promoter segment;
3. a splice signal segment;
- 15 4. a foreign DNA segment;
5. a poly adenylation segment;
6. the adenoviral packaging sequence;
7. the adenoviral ITR; and
8. all the plasmid DNA sequences necessary to
20 select and grow the plasmid in bacteria as well as mammalian tissue culture.

All references, including publications and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and
25 specifically indicated to be incorporated by reference and were set forth in its entirety herein.

While this invention has been described with
emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that the preferred
30 embodiments may be varied. It is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: GENVEC, Inc.
- (ii) TITLE OF INVENTION: COMPLEMENTARY ADENOVIRAL VECTOR SYSTEMS AND CELL LINES
- (iii) NUMBER OF SEQUENCES: 4
- (iv) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (v) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: WO PCT/US95/07341
 - (B) FILING DATE: 07-JUN-1995
 - (C) CLASSIFICATION:
- (vi) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 258416
 - (B) FILING DATE: 10-JUN-1995

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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32

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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34

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48

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCCGCCTCAT CCGCTTTT

18

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCGGAATTCC ACCATGGCGA GTCGGGAAGA GG

32

WHAT IS CLAIMED IS:

1. An adenoviral vector that is deficient in two
5 or more adenoviral regions.

2. The adenoviral vector of claim 1, wherein at
least one of the said two or more regions is selected
from the group of regions comprising the E1, E2, E3, and
10 E4 regions of the adenoviral genome.

3. The adenoviral vector of claim 1, wherein at
least one of the said two or more regions is selected
from the group of regions comprising the late regions of
15 the adenoviral genome.

4. The adenoviral vector of claim 2, wherein at
least one of the said two or more regions is selected
from the group of regions comprising the late regions of
20 the adenoviral genome.

5. The adenoviral vector of claim 1, wherein the
said two or more adenoviral regions include all the
adenoviral genes.

25

6. The adenoviral vector of claim 5, wherein said
adenoviral vector comprises adenoviral inverted terminal
repeats and one or more adenoviral promoters.

30 7. The adenoviral vector of claim 5, wherein said
adenoviral vector comprises adenoviral inverted terminal
repeats and a packaging signal.

8. The adenoviral vector of claim 1, wherein said
35 adenoviral vector only propagates in a complementing cell
line.

9. The adenoviral vector of claim 8, wherein said adenoviral vector only propagates in a complementing cell line as a result of the modification of adenoviral inverted terminal repeats or packaging signal.

5

10. A cell line that complements an adenoviral vector of claim 1, wherein at least two of said regions are essential for propagation.

10

11. A cell line that complements an adenoviral vector of claim 2, wherein at least two of said regions are essential for propagation.

12. A cell line that complements an adenoviral
15 vector of claim 3, wherein at least two of said regions are essential for propagation.

13. A cell line that complements an adenoviral
20 vector of claim 4, wherein at least two of said regions are essential for propagation.

14. A cell line that complements an adenoviral
vector of claim 5, wherein at least two of said regions are essential for propagation.

25

15. A cell line that complements an adenoviral vector of claim 6, wherein at least two of said regions are essential for propagation.

16. A cell line that complements an adenoviral
30 vector of claim 7, wherein at least two of said regions are essential for propagation.

17. A cell line that complements an adenoviral
35 vector of claim 8, wherein at least two of said regions are essential for propagation.

18. A cell line that complements an adenoviral vector of claim 9, wherein at least two of said regions are essential for propagation.

5 19. A cell line selected from the group consisting of cell lines designated as 293/E4, 293/ORF-6, 293/E2A, and 293/E4/E2A.

20 20. A recombinant multiply deficient adenoviral vector of claim 1 comprising a foreign DNA.

21. The recombinant vector of claim 20, wherein said foreign DNA is the cystic fibrosis transmembrane regulator gene.
15

22. The recombinant vector of claim 20, wherein said recombinant vector is selected from the group consisting of Ad_{Gv}.10, Ad_{Gv}.11, Ad_{Gv}.12, and Ad_{Gv}.13.

20 23. The recombinant vector of claim 22, wherein said recombinant vector is selected from the group consisting of Ad_{Gv}CFTR.10, Ad_{Gv}CFTR.11, Ad_{Gv}CFTR.12, and Ad_{Gv}CFTR.13.

25 24. A recombinant multiply deficient adenoviral vector of claim 1 comprising a DNA sequence that expresses in a mammal a therapeutic or prophylactic agent.

30 25. The recombinant multiply deficient adenoviral vector of claim 24, wherein said therapeutic agent is an antisense molecule selected from the group consisting of mRNA and a synthetic oligonucleotide.

35 26. The recombinant multiply deficient adenoviral vector of claim 24, wherein said prophylactic agent is a

DNA sequence that is expressed in a mammal as a polypeptide that elicits an immune response to said polypeptide.

5 27. A method of gene therapy comprising the administration to cells from a patient in need of gene therapy a therapeutically effective amount of a recombinant multiply deficient adenoviral vector of claim 20.

10

 28. A method of gene therapy comprising the administration to cells from a patient in need of gene therapy a therapeutically effective amount of a recombinant multiply deficient adenoviral vector of claim 21.

15

 29. A method of gene therapy comprising the administration to cells from a patient in need of gene therapy a therapeutically effective amount of a recombinant multiply deficient adenoviral vector of claim 22.

 30. A method of gene therapy comprising the administration to cells from a patient in need of gene therapy a therapeutically effective amount of a recombinant multiply deficient adenoviral vector of claim 23.

 31. The method of claim 28, wherein the recombinant multiply deficient adenoviral vector is administered to the lungs of said patient.

 32. The method of claim 30, wherein the recombinant multiply deficient adenoviral vector is administered to the lungs of said patient.

35

33. A method of therapy comprising the administration to cells from a patient in need of therapy a therapeutically effective amount of a recombinant multiply deficient adenoviral vector of claim 1
5 comprising a DNA sequence that is expressed as a therapeutic agent.

34. The method of claim 33, wherein said therapeutic agent is an antisense molecule selected from
10 the group consisting of mRNA and a synthetic oligonucleotide.

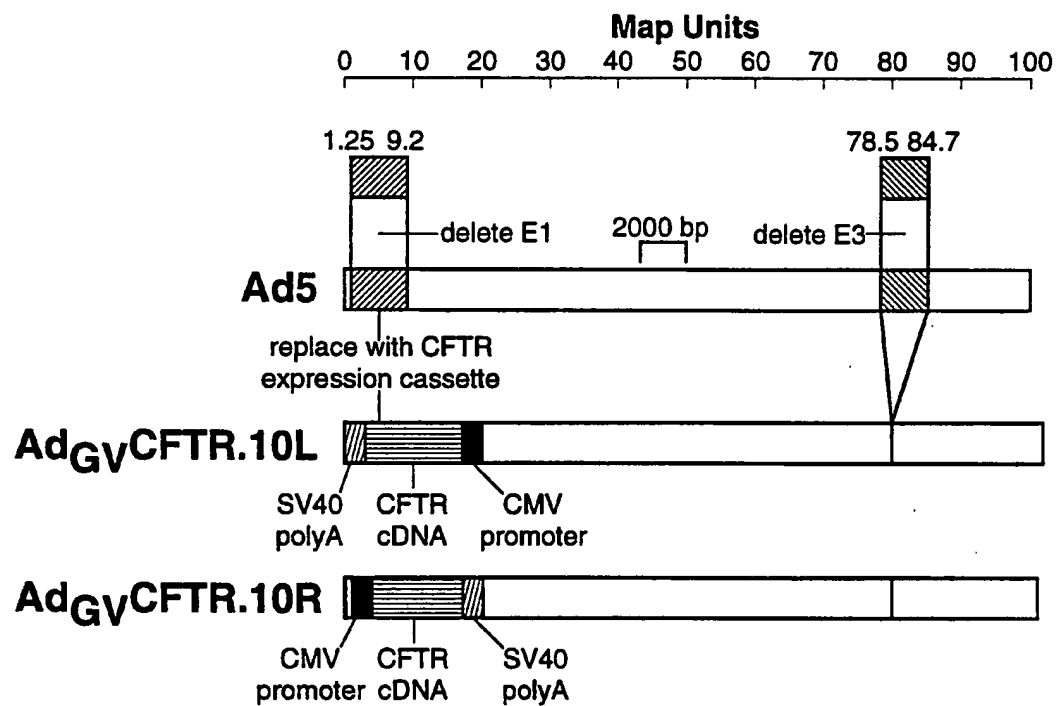
35. A method of vaccination comprising the administration to a patient in need of vaccination an
15 immunity-inducing effective amount of a recombinant multiply deficient adenoviral vector of claim 1 comprising a DNA sequence that is expressed as a polypeptide that elicits an immune response to said polypeptide.

20

36. A method of testing expression or toxicity of a foreign DNA-containing recombinant multiply deficient adenoviral vector of claim 20 in the cells of a patient in need of gene therapy, which method comprises (1)
25 removing and placing in culture an aliquot of target cells from said patient, (2) contacting said cells with said vector, and (3) measuring expression of said foreign DNA and vitality of said cultured target cells.

30 37. A method of testing foreign DNA expression upon transfection into a target cell, which method comprises (1) selecting a target cell, (2) contacting said target cell with a foreign DNA-containing recombinant multiply deficient adenoviral vector of claim 20, and (3)
35 measuring expression of said foreign DNA in said target cell.

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**FIG.1**

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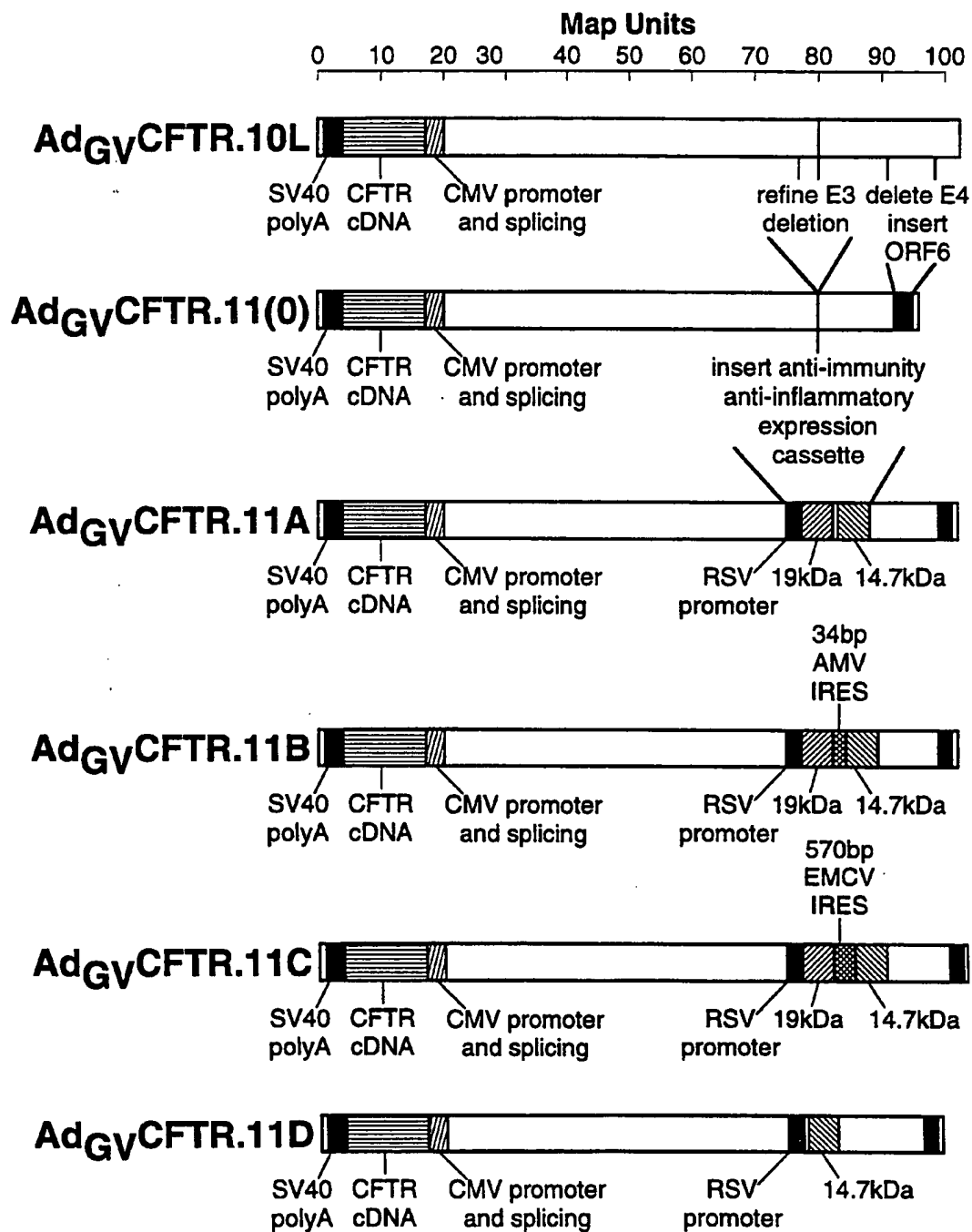
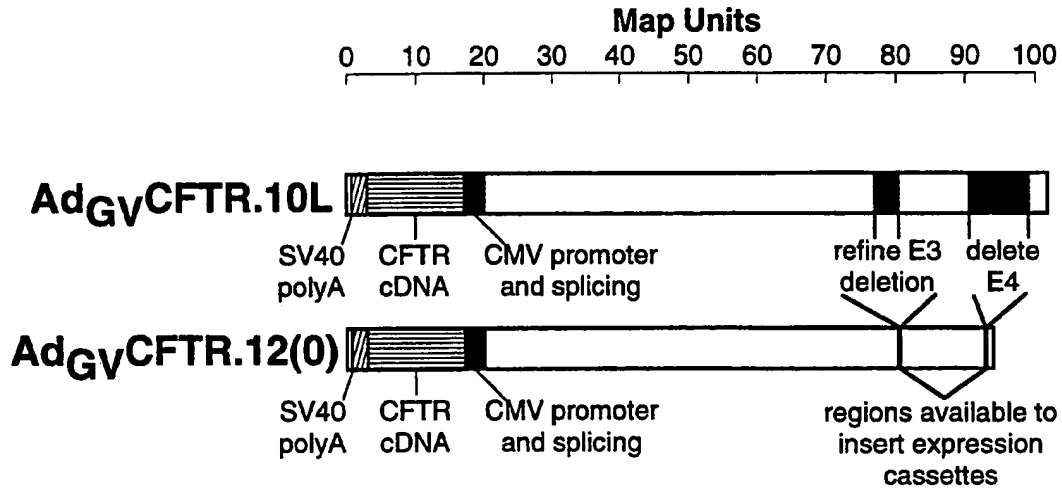
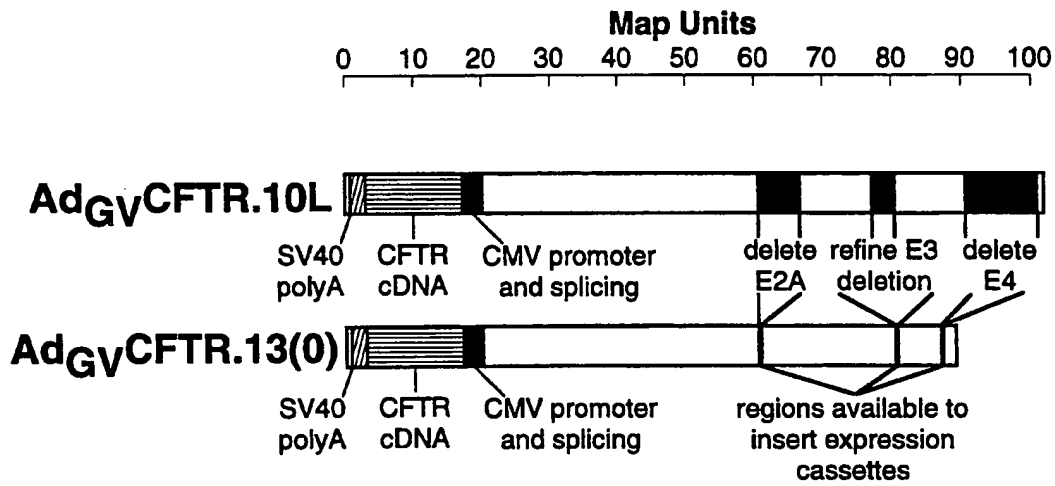


FIG.2

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**FIG.3****FIG.4**

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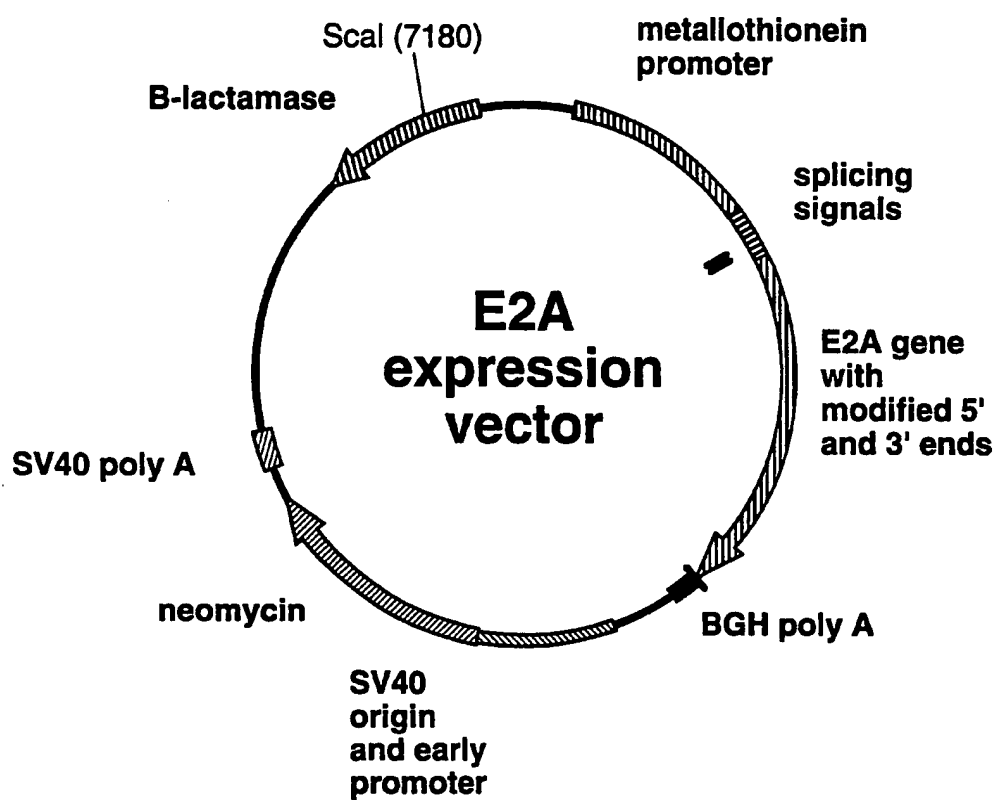


FIG. 5

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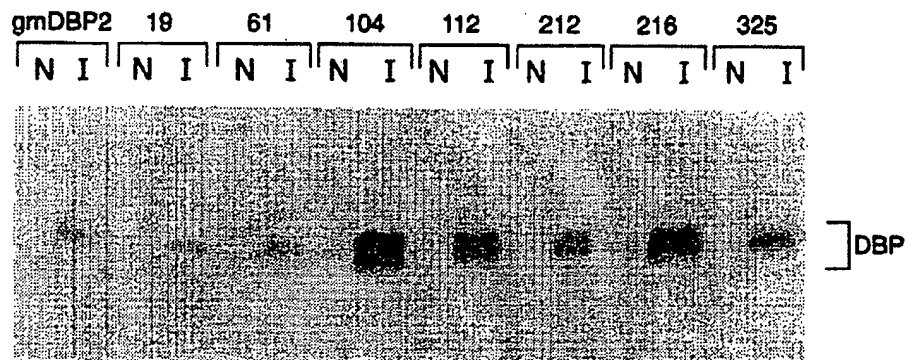


FIG. 6

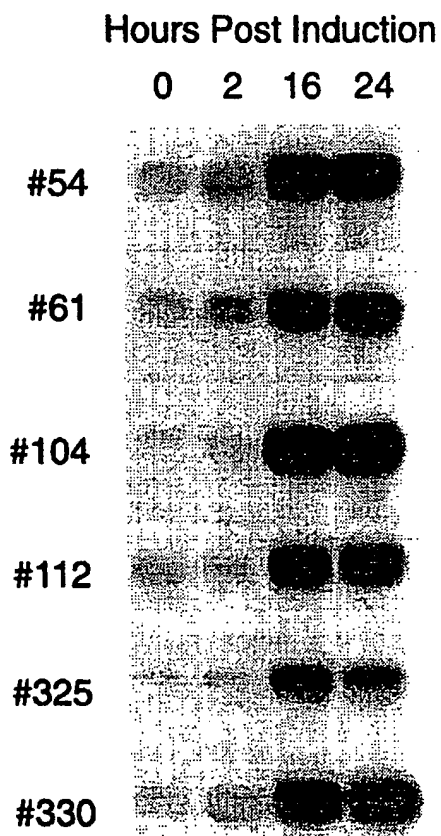


FIG. 7

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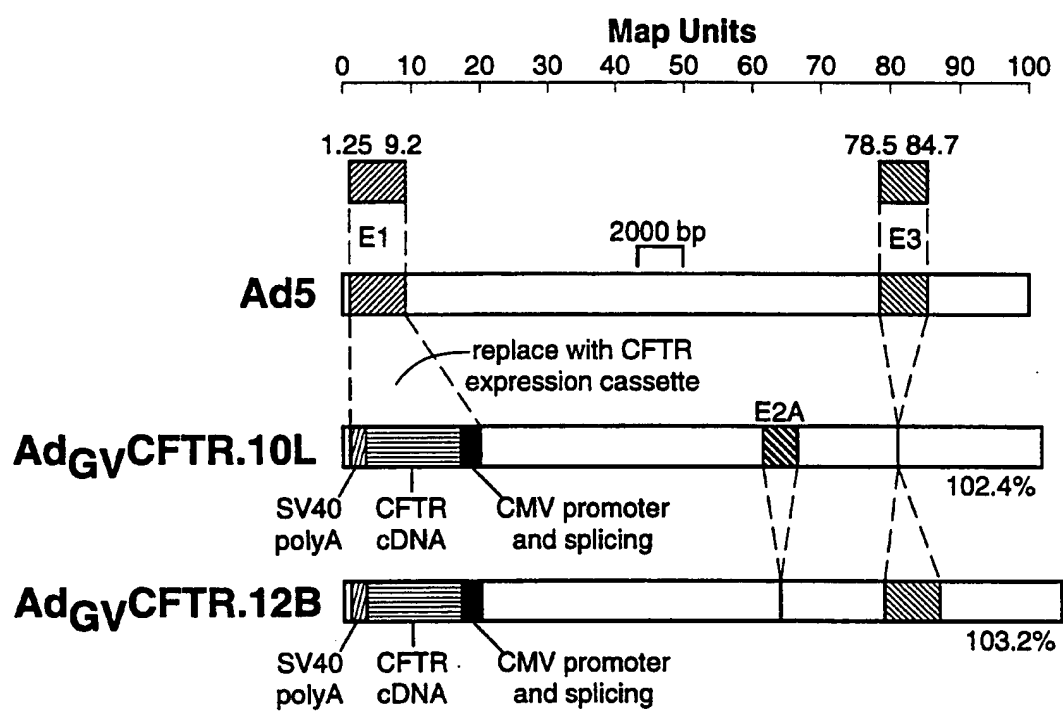


FIG.8

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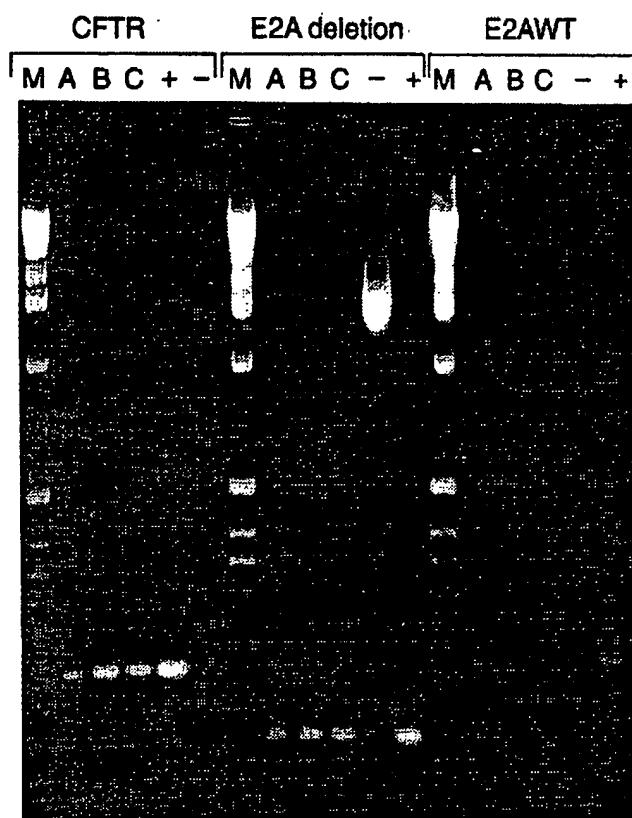


FIG. 9

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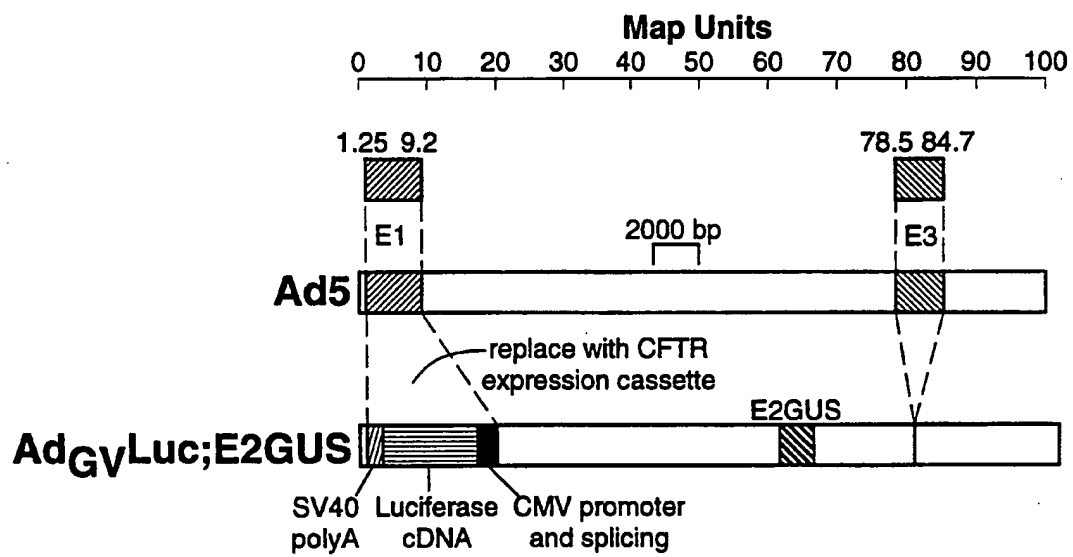
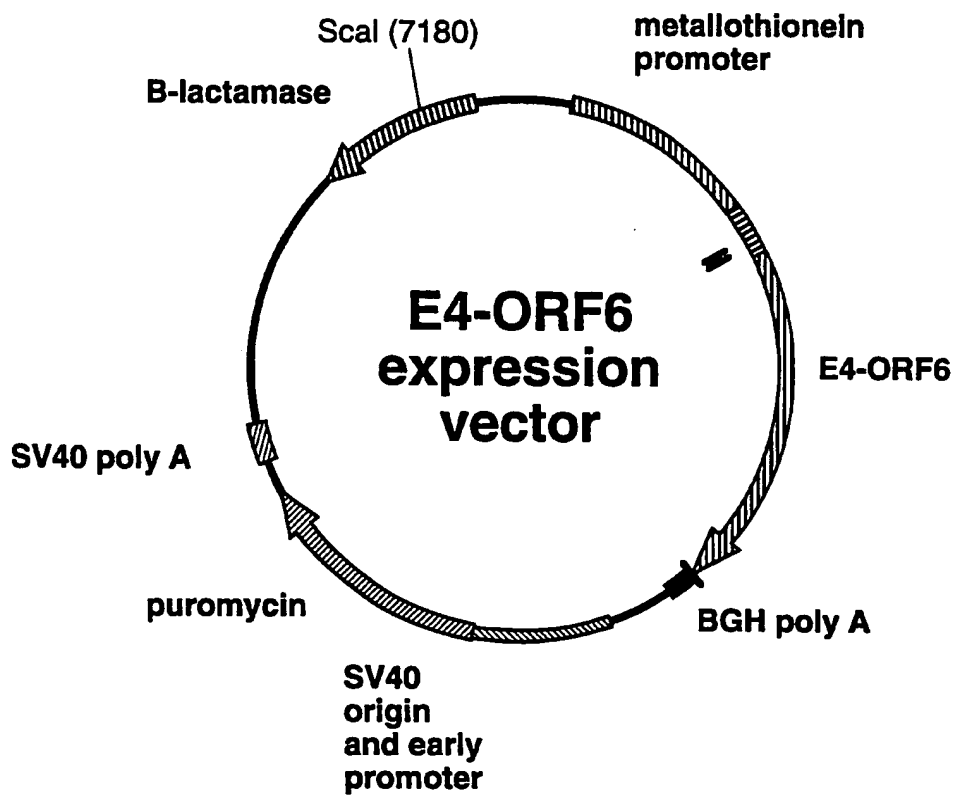


FIG.10

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**FIG. 11**

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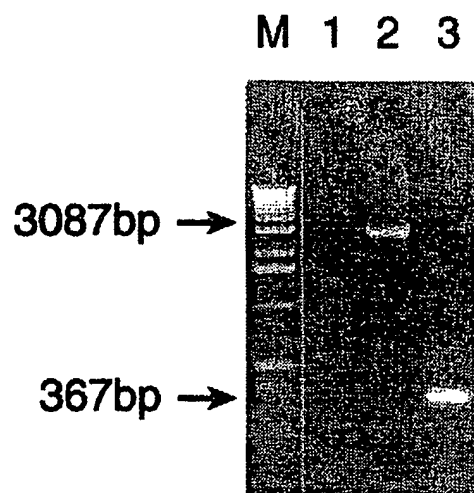
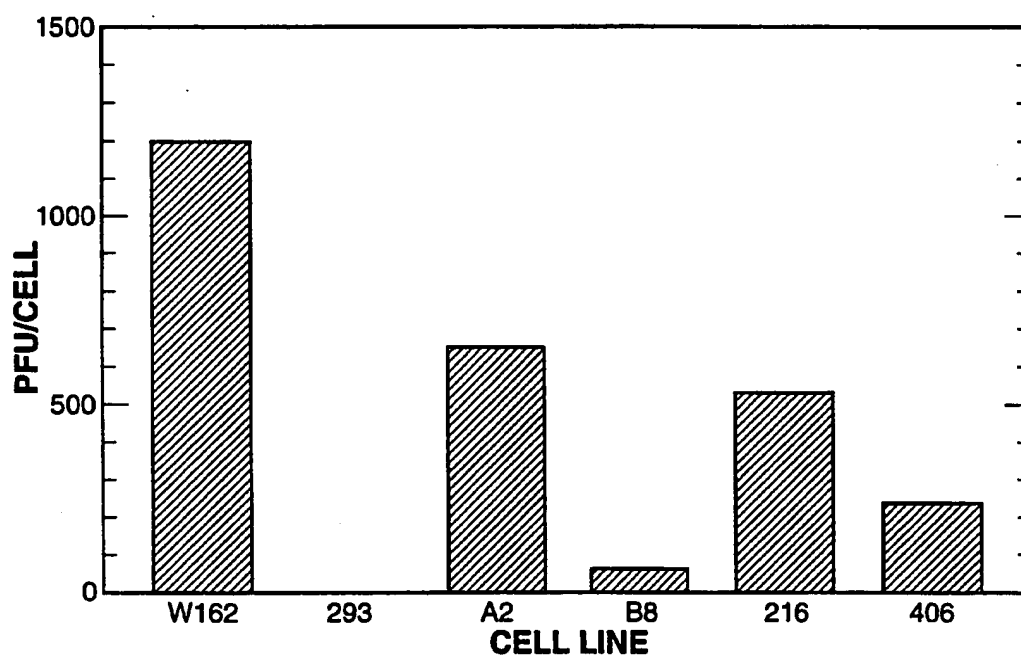


FIG. 12

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**FIG. 13**

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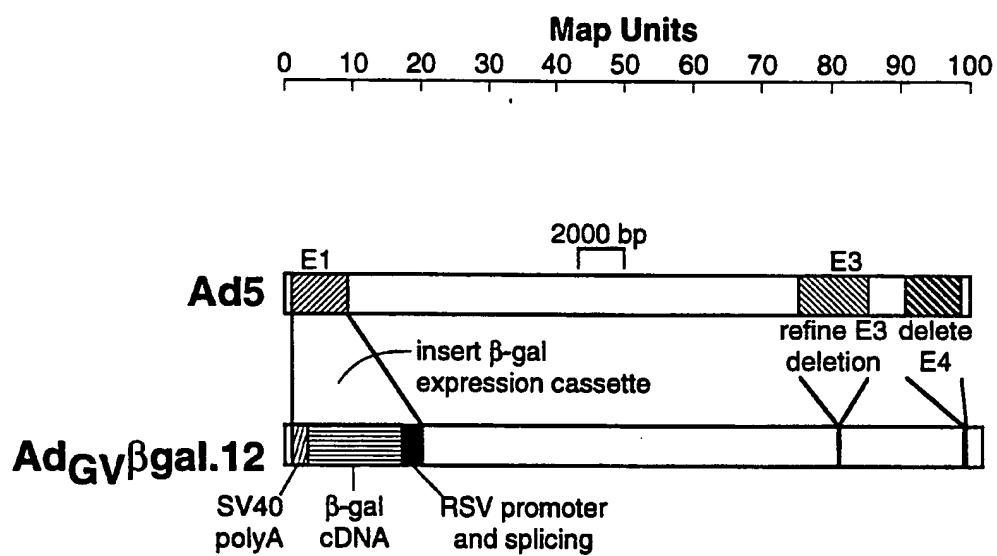


FIG.14

INTERNATIONAL SEARCH REPORT

Intern: Application No
PCT/US 95/07341

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C07K14/075 C12N15/34 A61K48/00 C07K14/47
C12N5/10 C12N7/01 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 08026 (RHONE-POULENC RORER) 14 April 1994 see claims 1-3,10,19-22 ---	1,2,20, 24,25, 27,33, 36,37
X	WO,A,94 12649 (GENZYME CORPORATION) 9 June 1994 see claims 5,8,12,16,17,20,24,25 ---	1,2,5,7, 8,20,21, 23,27, 28,30-33
X	WO,A,94 11506 (ARCH DEVELOPMENT CORPORATION) 26 May 1994 see claims 4,13-16,26 --- -/--	1,2,20, 24,25, 27,33,34

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

7 September 1995

Date of mailing of the international search report

25.09.95

Name and mailing address of the ISA

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Authorized officer

Chambonnet, F

INTERNATIONAL SEARCH REPORT

Intern: 1 Application No
PCT/US 95/07341

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,94 28152 (TRANSGENE S.A.) 8 December 1994 see the whole document ----	1-5,7,8, 10-14, 16,17, 20,21, 24,27, 28,31,33
P,X	WO,A,94 28938 (THE REGENTS OF THE UNIVERSITY OF MICHIGAN) 22 December 1994 see claims ----	1,2,20, 21,24, 27,28, 30,33,34
P,X	FR,A,2 707 664 (CNRS) 20 January 1995 see the whole document ----	1-4,8, 10-13, 17,20, 24-27, 33,35
P,X	WO,A,95 00655 (MCMASTER UNIVERSITY) 5 January 1995 see the whole document -----	1,2,10, 20,24, 27,33,34

INTERNATIONAL SEARCH REPORT

national application No.

PCT/US 95/07341

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 27-35
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 27-35 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. Application No

PCT/US 95/07341

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9408026	14-04-94	AU-B- 4818093 CA-A- 2145535 EP-A- 0669987 FI-A- 951404 NO-A- 951121	26-04-94 14-04-94 06-09-95 24-03-95 23-03-95
WO-A-9412649	09-06-94	AU-B- 5734994 CA-A- 2145641	22-06-94 09-06-94
WO-A-9411506	26-05-94	AU-B- 5609394 EP-A- 0668913	08-06-94 30-08-95
WO-A-9428152	08-12-94	FR-A- 2705686 AU-B- 6850394 CA-A- 2141212 EP-A- 0652968	02-12-94 20-12-94 08-12-94 17-05-95
WO-A-9428938	22-12-94	NONE	
FR-A-2707664	20-01-95	AU-B- 7264694 CA-A- 2144040 EP-A- 0667912 FI-A- 951138 WO-A- 9502697 NO-A- 950939	13-02-95 26-01-95 23-08-95 13-04-95 26-01-95 10-03-95
WO-A-9500655	05-01-95	AU-B- 7118494	17-01-95

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